IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Habener et al.	Examiner:	M.A. Belyavskyi
Serial No.:	09/963,875		
Filed:	September 26, 2001	Group Art Unit:	1644
Titled:	Stem Cells of the Islets of Langerhans and Their Use In Treating Diabetes Mellitus	Conf. No.:	9674

DECLARATION UNDER 37 CFR 1.131 BY JOEL F. HABENER, M.D.

I declare:

1.   I, Joel F. Habener hold an M.D. degree from the University of California, Los Angeles. I received my M.D. degree in 1965. My current positions are Investigator, Howard Hughes Medical Institute at the Massachusetts General Hospital, Associate Physician at the Massachusetts General Hospital and Professor of Medicine at Harvard Medical School. I have held the position of Howard Hughes Investigator since 1976. I have held the position of Associate Physician since 1989. I have held the position of Professor of Medicine at Harvard Medical School since 1989. Previously, I held the position of Associate Professor of Medicine at Harvard Medical School from 1975-1988. I am an inventor of the above-referenced patent application.

2.   I have read the Office Action dated December 23, 2003, filed in the above-referenced patent application and understand that the Examiner has rejected claims 39-43 and 74 for alleged lack of novelty in view of Zulewski et al. March 2001, Diabetes, 50:521. The Examiner has

stated at page 4 of the Office Action that "Zulewski et al. teach an isolated nestin-positive human pancreatic stem cell[s] that are not a neural stem cell[s] that can differentiate to form insulin-producing cells…[w]hile Zulewski et al., do not specifically teach that these cells are GLP-1R positive cells, said cells would inherently be GLP-1R-positive cells, since the cell population taught by Zulewski et al. is identical to that claimed in the instant application."

3.   I initially conceived of the idea that GLP-1 would stimulate neogenesis of beta cells in June, 1986. This idea was the basis for California Biotechnoloy Inc.'s (Cal Biochem) establishment of a subsidiary company called Metabolic Biosystems, Inc. (Meta Bio Inc.) for which I served as a consultant for several years (see Exhibit C).

4. In about 1996 my laboratory began to investigate the idea that GLP-1 stimulated neogenesis of beta cells. By July 1997, my laboratory had demonstrated that GLP-1 stimulates the neogenesis of pancreatic beta cells (see Exhibit C).

5. The concept of GLP-1 stimulation of neogenesis of beta cells was discussed with Doris Stoffers and Josephine Egan at the International Congress of Endocrinology/ADA meetings in San Francisco in June 1996 (see Exhibit C).

6. My laboratory began mouse and rat experiments to address whether GLP-1 stimulated neogenesis of beta cells at the time of the ADA meeting in Boston in June 1997. These experiments are described in an NIH grant application filed on February 27, 1997 (see Exhibit D) and in an invention disclosure submitted to MGH CSRL on October 28, 1997 (see Exhibit E). Attached herein are laboratory notes from my Senior Technician, Heather Hermann, from July 1987, that document experiments addressing stimulation of growth of beta cells in vitro with GLP-1 (See Exhibit F).

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7. The idea that GLP-1 stimulated the differentiation of new beta cells was premised on the concept that the progenitor cells expressed GLP-1 receptors.

I hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

May 11, 2004
Date

Joel F. Habener

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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2.   I have read the Office Action dated December 23, 2003, filed in the above-referenced patent application.

3.   My laboratory has developed a method for isolating a stem cell from a pancreatic islet of

Langerhans that includes the steps of: (a) removing a pancreatic islet from a donor, (b) removing cells from said pancreatic islet wherein said islet comprises a plurality of cell types comprising stem cells; and (c) separating said stem cells from said plurality of cells.

4.   Isolated human pancreatic islets were obtained through the JDRF Human Islet Distribution Program from the following centers: The Joslin Diabetes Center, Boston, the Northwest Tissue Center Islet and Cell Processing Laboratory, Seattle, and the Islet Distribution Center at the Diabetes Research Institute, Miami. Culture conditions were as follows. Single cell suspensions of Human pancreatic islet preparations were made by digestion with 5mg/ml Trypsin in PBS at 37°C and passage through a glass pipette. Viable cells were counted by Trypan Blue exclusion and seeded at 10,000 cells/cm^2 on tissue culture treated plastic dishes (Corning, Corning, NY). In initial experiments cell expansion was done in RPMI 1640 (11 mmol/l Glucose) (Invitrogen, Carlsbad, CA) with 10 mmol/l Hepes buffer, 1 mmol/l sodium pyruvate, 10% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA), 25ng/ml EGF, 20ng/ml bFGF and 1x penicillin/streptomycin. In later experiments CMRL 1066 medium (5.5 mmol/l Glucose) with 10% FBS, 1x penicillin/streptomycin, 100ng/ml beta nerve growth factor (β-NGF; R&D Systems, Minneapolis, MN), and 25ng/ml EGF was used. 24 to 48 hours after seeding, dead cells were removed by a media change and one wash with PBS. Thereafter, cells were expanded for 10-14 days until they reached confluence and medium was changed every 3 days.

According to this protocol, upon culture initiation only a few cells (less than 10%) attached to the dish and began to proliferate whereas the majority of cells did not attach to the dish and were washed off and discarded with the subsequent 2-3 changes of the culture media. Over 10-14 days of culture, the cells reached confluency. At this time, the levels of insulin mRNA in the cultured cells had markedly decreased compared to that of initial islets.

Immunostaining revealed only an occasional insulin-positive cell within the monolayer of expanded cells.

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The resulting expansion cultures of progenitor cells contain at least two phenotypically distinct cell types, those that express nestin and vimentin and those that express epithelial markers cytokeratin 19 and E-cadherin, as detected by immunofluorescent staining as described below.

Expansion phase cells were grown on tissue culture treated plastic slides (Nalge Nunc, Naperville, IL). For immunostaining cells were fixed with PBS/4% paraformaldehyde for 10 minutes at room temperature (RT). Slides were blocked with normal donkey serum in PBS/0.1%Triton for 1 hour at RT, incubated with primary antibodies over night at 4°C, washed, incubated with Cy2/Cy3 labeled secondary antibodies for 1 hour at RT, washed again and mounted. In vitro generated cell clusters were embedded in a fibrin clot prior to fixation. Clotting was achieved by mixing solutions of human fibrinogen (80 mg/ml in PBS, Sigma, St. Louis, MO) and human thrombin (50 units/ml in 40 mmol/l $CaCl_2$, Sigma). Clots were fixed in 10% buffered formalin, dehydrated, embedded in paraffin and cut into 4μm sections. Sections were dewaxed in xylene, hydrated, boiled for 10 minutes in a microwave oven in 10mmol/l sodium citrate, pH 6 for antigen retrieval and stained as described above. Nuclei were counterstained with DAPI. Antibodies used were guinea pig anti-insulin (1:2,000), guinea pig anti-glucagon (1:2,000), rabbit anti-somatostatin (1:2,000), and guinea pig anti-pancreatic polypeptide (1:2,000; all from Linco, St. Charles, MS), rabbit anti human nestin (1:200, Chemicon, Temecula, CA), mouse anti vimentin (1:100, Signet, Dedham, MA) mouse anti smooth muscle actin (1:100, Sigma), mouse anti cytokeratin 19 (1:100, Sigma), mouse anti keratin (1:100, Chemicon), mouse anti desmin (1:100, Sigma).

Immunostaining revealed that expansion cultures consisted of two major types of cells: E-cadherin/cytokeratin 19 (CK19) positive epithelial cells growing in patches and vimentin/nestin-positive spindle-shaped cells growing separately from each other. Many of the spindle-shaped cells also co-expressed smooth muscle actin. Occasional cells with epithelial characteristics (E-cadherin and CK19-positive) also stained positive for nestin (See Figure 1A-F, attached).

The two major populations of cells are easily separated based on differences in their

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morphologies. The nestin/vimentin positive spindle shaped fibroblatoid cells are markedly different from that of the E-cadherin/CK19 positive flat, cuboidal epithelial-like cells that are in patches. Under regular or phase contrast light microscopy, using low power, nestin/vimentin positive cells that are clearly separated from the E-cadherin/CK19 cells which are in distinct patches are selected. In certain embodiments, the nestin/vimentin positive cells are "cloned" by replating them and expanding them, multiple times.

The majority of the spindle shaped cells are nestin and vimentin positive as demonstrated by repeated immunohistochemical staining of expansion phase cultures.

Expansion phase cells were grown on tissue culture treated plastic slides (Nalge Nunc, Naperville, IL). For immunostaining cells were fixed with PBS/4% paraformaldehyde for 10 minutes at room temperature (RT). Slides were blocked with normal donkey serum in PBS/0.1%Triton for 1 hour at RT, incubated with primary antibodies over night at 4°C, washed, incubated with Cy2/Cy3 labeled secondary antibodies for 1 hour at RT, washed again and mounted. In vitro generated cell clusters were embedded in a fibrin clot prior to fixation. Clotting was achieved by mixing solutions of human fibrinogen (80 mg/ml in PBS, Sigma, St. Louis, MO) and human thrombin (50 units/ml in 40 mmol/l $CaCl_2$, Sigma). Clots were fixed in 10% buffered formalin, dehydrated, embedded in paraffin and cut into 4µm sections. Sections were dewaxed in xylene, hydrated, boiled for 10 minutes in a microwave oven in 10mmol/l sodium citrate, pH 6 for antigen retrieval and stained as described above. Nuclei were counterstained with DAPI.

Antibodies used were guinea pig anti-insulin (1:2,000), guinea pig anti-glucagon (1:2,000), rabbit anti-somatostatin (1:2,000), and guinea pig anti-pancreatic polypeptide (1:2,000; all from Linco, St. Charles, MS), rabbit anti human nestin (1:200, Chemicon, Temecula, CA), mouse anti vimentin (1:100, Signet, Dedham, MA) mouse anti smooth muscle actin (1:100, Sigma), mouse anti cytokeratin 19 (1:100, Sigma), mouse anti keratin (1:100, Chemicon), mouse anti desmin (1:100, Sigma).

5.   In view of the following, I believe that 0.2 to 5% of the cells of the pancreas are nestin-

positive cells.

To estimate the number of nestin-positive cells in a pancreas my laboratory overlayed a transparent grid over Figures 1D and 6C of Zulewski, H., Abraham, EJ, Gerlach, MJ, Daniel, PB, Moritz, W., Muller, B., Vallejo, M., Thomas, MK, and Habener, JF, *Diabetes*, 2001, **50**:521-533, counted the nestin positive cells (yellow) and divided by the total number of DAPI positve cells (blue). Using this methodology we calculated that approximately 10% of the cells are nestin positive. However, these figures depict highly selected fields chosen to maximize the number of nestin-positive cells in the figures. The section of the islet shown in Figure 1D has 25 nestin positive cells. Since some islets have one or two nestin positive cells while other islets have as many as 10-20 nestin positive cells, I estimate that the number of nestin-positive cells in the islets is an average of 5-10 cells or 2-4%, on average. Nestin- positive cells tend to gather in islets and around ducts. Since only 2-3% of the cells of the entire pancreas are islet and ducts, I estimate that based on the results of the above analysis, 0.2 to 0.4% of the total cells of the pancreas are nestin positive.

To estimate the number of nestin-positive cells in the pancreas my laboratory performed immunocytochemistries on pancreas sections (nestin antiserum diluted 1:500). The number of nestin-positive cells in four fields containing non-islet cells is 26 of 461 DAPI-stained nuclei (5.6%), 23 of 442 DAPI-stained nuclei (5.2%), 14 of 408 DAPI-stained nuclei (3.4%) and 12 of 428 DAPI-stained nuclei (2.8%). The contribution of nestin-positive cells from the non-islet cells of the pancreas is therefore 4.25%, the average value for these four fields. The number of nestin-positive cells in four fields

containing only islets (insulin staining was used to identify the islet borders) is 19 of 375 DAPI-stained nuclei (5.1%), 21 of 234 DAPI-stained nuclei (9.0%), 15 of 243 DAPI-stained nuclei (6.1%) and 6 of 132 DAPI-stained nuclei (4.58%). The average value for these four fields is 6.2%. The islets make up approximately 2% of the total pancreas tissue. The contribution of nestin positive cells from the islets is 2% of 6.2% or 0.124%. In view of the above, the total number of nestin-positive cells, as determined by the immunocytochemical method described above, is approximately 4.4%.

It is my belief that in view of the above, one of skill in the art would accept that the percentage of nestin-postive cells in the pancreas is in the range of 0.2 to 5%, as determined by the two independent methods described hereinabove.

I hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

June 17, 2004
Date

Joel F. Habener
Joel F. Habener

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Insulinotropic Hormone Glucagon-Like Peptide-1 Differentiation of Human Pancreatic Islet-Derived Progenitor Cells into Insulin-Producing Cells

ELIZABETH J. ABRAHAM, COLIN A. LEECH, JULIA C. LIN, HENRYK ZULEWSKI, AND JOEL F. HABENER

Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02114

Glucagon-like peptide-1 (GLP-1) is an intestinal incretin hormone, derived from the processing of proglucagon, that exerts insulinotropic actions on insulin-producing pancreatic islet β-cells. Recently GLP-1 was shown to stimulate the growth and differentiation (neogenesis) of β-cells and appears to do so by inducing the expression of the homeodomain protein IDX-1 (islet duodenum homeobox-1; also known as PDX-1, pancreatic and duodenal homeobox gene; and as IPF-1, insulin promoter factor), which is required for pancreas development and the expression of β-cell-specific genes. Earlier we identified multipotential progenitor cells in the islet and ducts of the pancreas, termed nestin-positive islet-derived progenitor cells (NIPs). Here we report the expression of functional GLP-1 receptors on NIPs and that GLP-1 stimulates the differentiation of NIPs into insulin-producing cells. Furthermore, confluent NIP cultures express the proglucagon gene and secrete GLP-1. These findings suggest a model of islet development in which pancreatic progenitor cells express both GLP-1 receptors and proglucagon with the formation of GLP-1. Locally produced GLP-1 may act as an autocrine/paracrine developmental morphogen on receptors on NIPs, resulting in the activation of IDX-1 and the expression of the proinsulin gene conferring a β-cell phenotype. GLP-1 may be an important morphogen both for the embryonic development of the pancreas and for the neogenesis of β-cells in the islets of the adult pancreas. (Endocrinology 143: 3152–3161, 2002)

T HE PREVALENCE of diabetes mellitus is increasing throughout the world. Diabetes is caused to a large extent by a reduction in the fully functioning mass of insulin-producing β-cells that reside within the islets of Langerhans in the pancreas. As a consequence of a reduced mass of pancreatic β-cells, the amounts of insulin produced are insufficient to meet the body's needs, and hyperglycemia ensues (1, 2). Although recent studies indicate that islet transplantation may be a cure for diabetes (3), the availability of pancreata as a source for islet transplantation is severely limited. Therefore, it will be necessary to develop alternative sources of islet tissue. One such source may be progenitor cells that can be expanded ex vivo, differentiated into islet tissue, and transplanted.

The glucagon gene encodes a multifunctional proglucagon that is differentially processed by prohormone convertases 1 and 2 in the pancreas and the intestine. In the α-cells of the pancreas, the major product of proglucagon processing is glucagon, although small amounts of glucagon-like peptide-1 (GLP-1) are produced, whereas in intestinal L cells the major proglucagon-derived products are GLP-1 and GLP-2 (4, 5). However, in streptozotocin-induced diabetic rats there is a robust increase in pancreatic prohormone convertases 1 and 2, resulting in a 2-fold increase in the ratio of amidated GLP-1 to total glucagon immunoreactivity (6), indicating that

GLP-1 may play a role in regeneration of β-cell mass in a diabetic animal model. GLP-1 binds to specific G protein-coupled receptors on pancreatic β-cells to stimulate insulin secretion via cAMP-dependent pathways (4, 5). When administered to diabetic mice, GLP-1 lowers blood glucose levels and stimulates insulin secretion (7–10). In addition, GLP-1 increases β-cell mass by inducing the differentiation and neogenesis of ductal progenitor cells into islet endocrine cells (8–12). The antidiabetogenic potential of GLP-1 is currently under investigation and shows promise as a therapeutic agent in the treatment of type 2 diabetes (13).

Recently, we identified a distinct population of cells in pancreatic islets and ducts that expresses nestin (14). These nestin-positive islet-derived progenitor cells (NIPs), isolated from adult pancreatic islets, can differentiate in culture into cells with pancreatic exocrine, endocrine, and hepatic phenotypes. We hypothesized that GLP-1 receptors (GLP-1R) must be present on NIPs and that binding of GLP-1 to its receptors on these cells results in activation of the transcription factor IDX-1, a master regulator of endocrine pancreas development (15, 16). IDX-1 then activates the expression of the insulin gene, resulting in a β-cell phenotype (8, 12).

Here we show the expression of GLP-1 receptors on NIPs and that GLP-1 functionally activates NIPs by virtue of their depolarization and resultant increase in intracellular calcium. Notably, the activation of NIPs by GLP-1 is paradoxically inhibited in conditions of high (20 mM) ambient glucose concentrations, unlike the direct glucose-dependent activation of β-cells by GLP-1. Further, we show that GLP-1 stimulates the differentiation of NIPs into a pancreatic en-

docrine phenotype that expresses the homeodomain protein IDX-1 and the hormones insulin, glucagon, and GLP-1.

Materials and Methods

Reagents

GLP-1-(7–36)amide was obtained from Sigma (St. Louis, MO). Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), leukemia inhibitory factor were obtained from Sigma and Chemicon International (Temecula, CA), respectively. B-27 supplement was obtained from Life Technologies, Inc. (Gaithersburg, MD).

Isolation and culture of NIPs

Human islet tissue was obtained from the Juvenile Diabetes Research Foundation Center for Islet Transplantation, Harvard Medical School (Boston, MA), and the Diabetes Research Institute, University of Miami School of Medicine (Miami, FL). NIPs were isolated as described previously (14). Briefly, islets were washed and cultured in RPMI 1640 medium containing serum, 11.1 mM glucose, antibiotics, sodium pyruvate, β-mercaptoethanol, and growth factors. Within several days, nestin-positive cells grew out from islets. These cells were cloned and expanded in medium containing 20 ng/ml each of bFGF and EGF. In some instances, long-term passaged cells were maintained in 1000 U recombinant human leukemia inhibitory factor. For differentiation, NIPs were incubated with GLP-1 in the absence of serum, and fresh GLP-1 was added every 48 h without changing the medium. In some experiments differentiation was achieved by culturing NIPs in cell culture medium containing B-27 [DMEM/F-12 (1:1), B-27, bFGF, EGF, and antibiotics] as described by Toma *et al.* (17) for the culture of skin-derived precursors and for the differentiation of mouse embryonic stem cells (18). Similar to the skin precursors (17), NIPs cultured in B-27 medium generated spherical clusters of cells that were collected, centrifuged, and replated onto laminin-coated 48-well plates [BD Biosciences, Bedford, MA] and cultured in the B-27-supplemented medium now containing 10 nM GLP-1 and no other added growth factors, *i.e.* bFGF and EGF were absent.

Antibodies

We used rabbit polyclonal antisera to rat IDX-1 (14) and rat GLP-1 receptor (19), which cross-reacts with its human counterpart. The rabbit antihuman nestin was a gift from Dr. C. Messam (NINDS, NIH, Bethesda, MD). Guinea pig antiinsulin and antiglucagon sera were obtained from Linco Research, Inc. (St. Charles, MO). Cy-3- and Cy-2-labeled secondary antisera were purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA).

Immunocytochemistry

Cells cultured on Lab-Tek chamber slides (Nunc, Naperville, IL) or gridded coverslips (Bellco Glass, Inc., Vineland, NJ) were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After several rinses in PBS, cells were permeabilized with methanol/Triton-X in some instances, blocked with normal donkey serum for 30 min, and incubated with primary antiserum or preimmune serrum at 4 C. The following day, cells were rinsed with PBS and incubated with secondary antisera (donkey antirabbit and donkey antiguinea pig) labeled with Cy-3 or Cy-2 for 1 h at room temperature. After several washes, coverslips containing cells were mounted onto slides in mounting medium (Vector Laboratories, Inc., Burlingame, CA). Fluorescence images were obtained using a Carl Zeiss (New York, NY) epifluorescence microscope equipped with an Optronics TEC-470 CCD camera (Optronics Engineering, Goleta, CA) interfaced with a Powermac 7100. IP lab Spectrum software (Signal Analytics, Vienna, VA) was used to acquire and analyze images.

RT-PCR

Total cellular RNA prepared from NIP cultures or human islets were reverse transcribed and amplified by PCR for 40–45 cycles as described previously (20). Oligonucleotides for the PCR were as follows: human GLP-1 receptor: forward, 5'-gtgtggcggccaattactac-3'; reverse, 5'-cttg-gcaagtctgcatttga-3'; and human glucagon: forward, 5'-atctggactccag-gcgtgcc-3'; reverse, 5'-agcaatggattccttggcag-3'. An RT-negative control was run for most samples. PCR cycling for glucagon was at 94 C for 1 min, followed by 94 C for 10 sec, 56 C for 10 sec, and 72 C for 1 min (40 cycles), followed by 72 C for 2 min. A hot start PCR was performed for human GLP-1 receptor (GLP-1R) as follows: 94.5 C for 5 min, followed by addition of Taq polymerase and subsequent cycling at 94 C for 10 sec, 54 C for 10 sec, and 72 C for 10 sec (45 cycles). Primer extension at 72 C was performed for an additional 2 min.

Intracellular calcium ([Ca^{2+}]$_i$) measurements

NIPs plated on gridded coverslips were loaded with fura-2 by incubation in standard extracellular saline (138 mM NaCl, 5.6 mM KCl, 2.6 mM CaCl$_2$, 1.2 mM MgCl$_2$, and 10 mM HEPES) containing 5.6 mM glucose and supplemented with 2% fetal bovine serum, 0.01% pluronic F-127, and 5 μM fura-2/AM. Cells were loaded for 90 min at room temperature, washed with standard extracellular saline, and then transferred to a Peltier temperature-controlled stage at 32 C. Human serum albumin (0.05%) was added as a carrier protein during experiments with GLP-1-(7–36)amide and Exendin-(9–39), a specific antagonist of GLP-1. Calcium measurements were taken at 0.25 Hz using an IonOptix (Milton, MA) imaging system. The grid location was noted, and fluorescence images of the cells were recorded for subsequent identification of the cells for immunohistochemical staining. The solution in the recording chamber was exchanged by a gravity-fed perfusion system.

RIA

Insulin levels in culture media were measured by an ultrasensitive RIA kit purchased from Linco Research, Inc. and Diagnostic Products (Los Angeles, CA). The detection level for the insulin assay was 8 pg/ml. GLP-1 levels in culture media were measured by a GLP-1-specific RIA that uses rabbit antiserum raised against the C terminus of GLP-1-(7–36)amide and does not cross-react with glucagon or proglucagon.

Transfections

A fragment of the rat insulin I gene promoter that spans nucleotides −410 to 49 bp was fused to the coding sequence of luciferase in the pxp2 basic vector to generate the insulin promoter-luciferase construct (INS-LUC) (21). The human insulin promoter factor-1 (IPF-1) cDNA was a gift from Henk-Jan Anstoot (Sophia Children's Hospital, Rotterdam, The Netherlands). This cDNA was transferred to a cytomegalovirus 5 promoter vector in our laboratory (22). The rat IDX cDNA was cloned previously in our laboratory (23). Adherent NIP cultures plated in 12-well dishes were transfected with 0.6 μg rat INS-LUC and/or 0.125 μg rat IDX-1 cDNA/well for 5 h in serum-free culture medium using Lipofectamine 2000 (2.5 μl/well; Life Technologies, Inc.). A filler plasmid DNA was used to bring the DNA concentration to 1 μg/well. Then, cells were exposed to test substances in medium supplemented with 10% serum. After 20–24 h, cells were lysed, and luciferase activity was measured using a luciferase assay kit (Promega Corp., Madison, WI) in a luminometer (Wallac, Inc., Gaithersburg, MD). These experiments were carried out in duplicate wells and repeated at least three times. In other instances, NIP cultures were plated onto 4-well Lab-Tek chamber slides and transfected with human IPF-1 cDNA (0.2 μg/well) using Geneporter (Life Technologies, Inc.). The following day, transfected cells were incubated with GLP-1 (1–10 nM) in serum-supplemented medium. After 3–4 d, cells were fixed with 4% paraformaldehyde and subjected to immunostaining for IDX-1 and insulin.

Western immunoblot

NIP cultures plated in 10-cm dishes were either transfected with human IPF-1 cDNA using Geneporter or were left untransfected. These cells were subsequently treated with or without 10 nM GLP-1 in serum-supplemented medium for 3–4 d. Then, nuclear extracts were prepared according to the Schreiber method (24), and equal amounts of proteins (20 μg) were loaded and electrophoresed on premade NuPAGE (Invitrogen, Carlsbad, CA) gels according to the manufacturer's recommendations. The proteins were transferred onto a nitrocellulose mem-

brane and subjected to an IPF-1 immunoblot procedure as described previously (23).

Results

GLP-1 receptor expression in NIPs

We examined human NIPs for the presence of GLP-1R by immunocytochemistry. Receptor immunoreactivity was detected in the majority of NIPs (>60%; Fig. 1A). To further confirm the immunocytochemical identification of GLP-1R in NIPs, we performed an RT-PCR of GLP-1 receptor mRNA prepared from NIP cells and detected the product of the correct size (346 bp) for the GLP-1R (Fig. 1B). Clonal variation in the relative amounts of GLP-1R mRNA between lines was seen; receptor expression was lower in some NIP clones than others (see clone 9 *vs.* 2 in Fig. 1B), but was undetectable in only a minority of clones. The expression of GLP-1 receptors in NIPs indicates the potential for GLP-1-mediated regulation of islet progenitor cell differentiation by GLP-1.

### Functional GLP-1 receptor signaling in NIPs

The application of GLP-1 -(7–36)amide to single isolated NIPs elevates $[Ca^{2+}]_i$. Cells were plated onto gridded coverslips to permit subsequent immunohistochemical staining of the same cells to test for nestin expression. All cells examined that increased $[Ca^{2+}]_i$ in response to GLP-1 were nestin positive. In contrast to β-cells prepared from adult human islets, in human NIPs, GLP-1 stimulated $[Ca^{2+}]_i$ at basal (5.6 mM; n = 45 cells) glucose, but had no effect on $[Ca^{2+}]_i$ in the presence of high (20 mM) glucose (Fig. 2A). These glucose-related effects on $[Ca^{2+}]_i$ responses in NIPs were reproduced by forskolin (Fig. 2B), suggesting that the effects of GLP-1 on NIPs are mediated via the activation of G_s and cAMP production, the same signaling pathway used by GLP-1 in adult islet-derived β-cells. However, the glucose dependence of GLP-1 in NIPs differs strikingly from that in adult β-cells, inasmuch as in adult β-cells the actions of GLP-1 are markedly directly dependent on glucose concen-

trations (25, 26). These findings suggest that the coupling of glucose signaling with cAMP signaling (25) in NIP progenitor cells is different from that in adult islet-derived β-cells. The pretreatment of single isolated NIPs with the peptide exendin-(9–39), a specific antagonist of GLP-1, prevents the increase in $[Ca^{2+}]_i$ mediated by GLP-1 (Fig. 2, C and D). These inhibitory effects of the GLP-1R antagonist exendin-(9–39) on $[Ca^{2+}]_i$ responses suggest that the same isoform of GLP-1R is expressed in NIPs as that expressed in β-cells. The increase in $[Ca^{2+}]_i$ mediated by GLP-1 on NIPs was inhibited by extracellular La^{3+} (5 μM), indicating that GLP-1 is activating $[Ca^{2+}]_i$ influx, consistent with its known role to depolarize β-cells (Fig. 2E). We demonstrate further that tolbutamide (100 μM) stimulates the $[Ca^{2+}]_i$ elevation in NIPs, indicating that NIPs must also express ATP-sensitive K^+ channels (Fig. 2F). These findings suggest that GLP-1 induces membrane depolarization and activation of voltage-dependent Ca^{2+} channels in NIPs, consistent with its known mechanism of action in β-cells. However, unlike its known actions in β-cells, the activation of ion channels in NIPs is inhibited by high (20 mM) glucose.

GLP-1 induces differentiation of NIPs into insulin-secreting cells

Previous studies demonstrated the insulinotropic actions of GLP-1 as well as its ability to stimulate β-cell neogenesis in partial pancreatectomized rats (9). Therefore, we determined whether GLP-1 would induce differentiation of human NIPs into insulin-secreting cells. As described previously (14), human islets were cultured in medium containing bFGF and EGF for 14 d. The majority of islets became degranulated, and a monolayer of cells grew out from them. Immunocytochemical analysis of such islet cultures showed that the outgrowing cells were mostly all nestin positive and insulin negative; just a few cells in the monolayer expressed insulin (Fig. 3A). NIPs were picked from these cultures and expanded in growth factor-supplemented medium (passage 1) for 3 d as described previously (14). In certain instances

FIG. 1. Expression of GLP-1R on pancreatic islet-derived stem/progenitor cells. A, NIPs (passages 6–8) plated on gridded coverslips were fixed and subjected to immunocytochemical detection with antiserum to GLP-1R (α-GLP-1R) (Cy-3, rendered intense *white* on modifed photomicrograph) or a preimmune serum control. Note the punctate fluorescence on the surface of the cell, typical of receptor aggregation. B, RT-PCR of RNA prepared from different clones of NIPs (passages 4–8) using oligonucleotide amplimers to human GLP-1R give the predicted 346-bp product, which was confirmed by Southern blotting. Human islet tissue was used as the positive control.
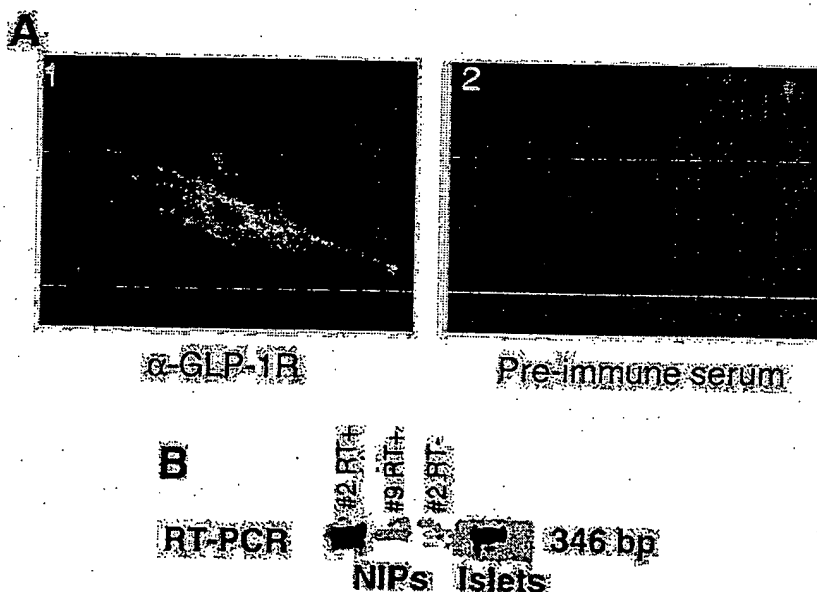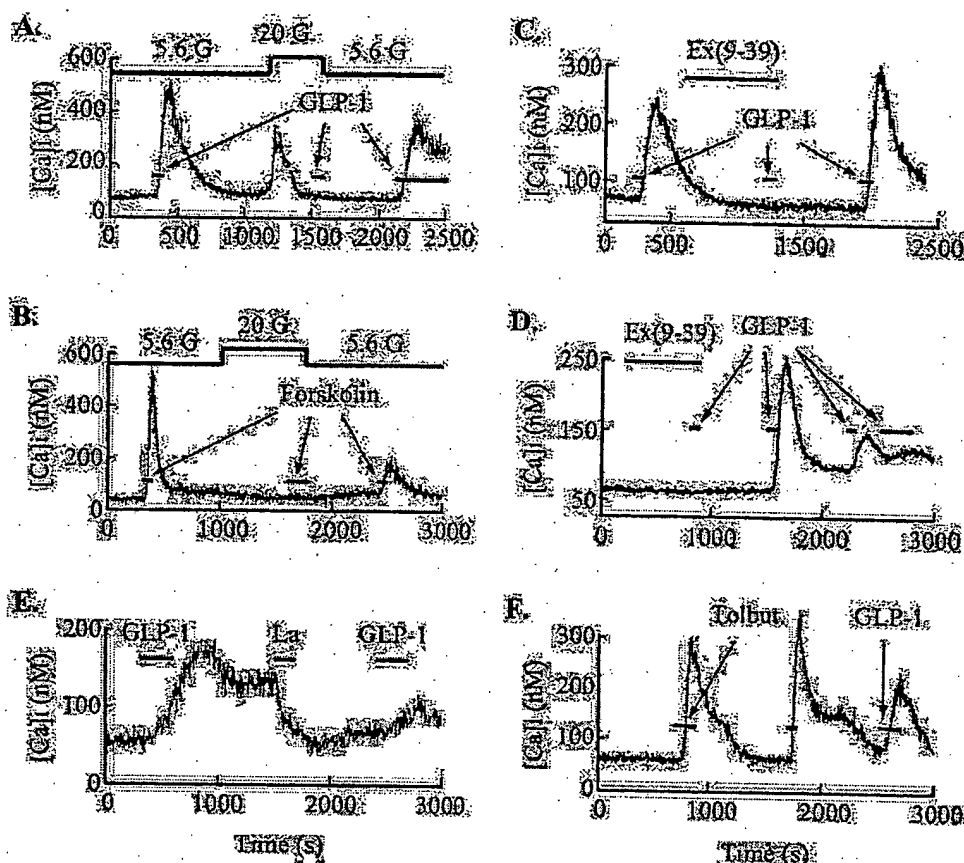
FIG. 2. GLP-1-(7–36)amide and tolbutamide stimulate $[Ca^{2+}]_i$ influx in NIPs. A, Fura-2-loaded cells bathed in 5.6 mM glucose show an increase in the $[Ca^{2+}]_i$ response to 10 nM GLP-1. Increasing extracellular glucose to 20 mM (20 G) also caused an increase in $[Ca^{2+}]_i$, but application of GLP-1 in 20 mM glucose failed to produce an additional $[Ca^{2+}]_i$ response. A third application of GLP-1 on returning to 5.6 mM glucose produced a $[Ca^{2+}]_i$ response. These effects of GLP-1 on $[Ca^{2+}]_i$ were observed in 45 different cells tested. B, The glucose-dependent effects of GLP-1 were reproduced by 10 mM forskolin, suggesting that the $[Ca^{2+}]_i$ elevation is mediated by the cellular cAMP level. C, The GLP-1-mediated increase in $[Ca^{2+}]_i$ was reversibly inhibited by 10 nM exendin-(9–39). This inhibitory effect was not due to receptor desensitization (D), because application of GLP-1 in the presence of the GLP-1 receptor antagonist exendin-(9–39) failed to produce a response, whereas subsequent applications of GLP-1 after washout of exendin-(9–39) produced repeated $[Ca^{2+}]_i$ elevations. E, The GLP-1-mediated increase in $[Ca^{2+}]_i$ is inhibited by 0.5 mM extracellular La^{3+}, an inhibitor of Ca^{2+} influx, suggesting that GLP-1 stimulates Ca^{2+} influx. F, NIPs bathed in 5.6 mM glucose were stimulated with 100 mM tolbutamide (Tolbut.) and responded to repeated applications of tolbutamide with increases in $[Ca^{2+}]_i$. Application of 10 nM GLP-1 also stimulated an increase in $[Ca^{2+}]_i$, suggesting that GLP-1 acts by depolarizing the cells. Shown in each panel are representative recordings from single NIPs (clone 006a) that respond to the above-mentioned test substances.

NIPs that were expanded for 3–5 d spontaneously expressed insulin. We find that at this stage of passage (30–40 cells/dish) the vast majority of NIPs were nestin positive and insulin negative (Fig. 3B). When NIP cultures were expanded for 7–12 d and then treated with GLP-1, a subset of cells became insulin positive (Cy-2; *green*) and nestin negative (Cy-3; *red*; Fig. 3, C and D). Incubation with exendin-(9–39), a specific antagonist of GLP-1, abolished the appearance of immunostaining for insulin (Fig. 3E). The cells treated with GLP-1 also changed their morphology, becoming more rounded and flattened (Fig. 3D *vs.* Fig. 3, C and E). The percentage of differentiated cell progeny by virtue of insulin staining varies from 5% or less to 30% depending on the particular cloned NIP culture tested. Treatment with exendin-4, a long-acting analog of GLP-1, induced a 2- to 3-fold increase in insulin secretion, as measured by RIA of the cell culture medium (Fig. 3G). The secretory response to exendin-4 was detected in 30% of the NIP clones tested. In some culture wells confluence

alone was sufficient to initiate the secretion of small amounts of insulin, indicating that cell confluence can induce differentiation to some extent.

Human NIPs that have been repeatedly passaged lose their ability to secrete insulin in response to GLP-1. However, a modification of the differentiation protocols of these cells can render them again responsive to GLP-1. In these modified experiments, long-term passaged NIPs (≤6 months) were cultured in medium supplemented with B-27, bFGF, and EGF for 4–6 d or more. Similar to skin-derived precursor stem cells (17), NIPs in this medium form floating clusters after 6 d of culture (Fig. 4A). For differentiation experiments, these clusters were collected, centrifuged, and plated on laminin-coated dishes in the B-27-supplemented medium now containing 10 nM GLP-1 but no growth factors (bFGF and EGF). A week later, cells grew out from the clusters, which were then fixed and subjected to immunocytochemistry for insulin and IDX-1.
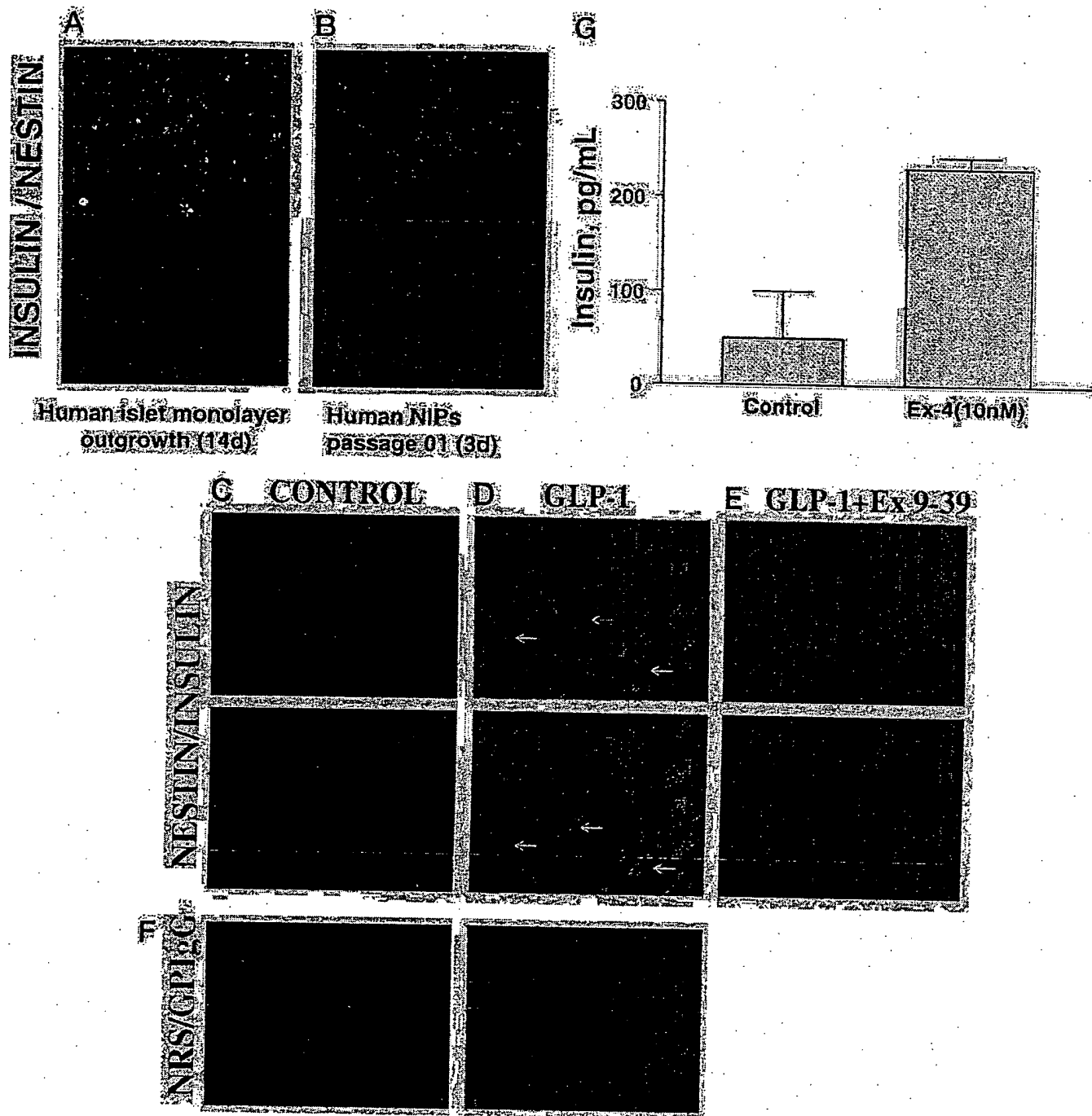
FIG. 3. GLP-1 induces differentiation of NIPs into insulin-producing cells. A, Human islets were cultured in growth medium containing bFGF and EGF for 14–18 d. The monolayer of cells that grew out from the islet was fixed with 4% formaldehyde and immunostained with antiserum for insulin (Cy-2, *green*) and nestin (Cy-3, *red*). The majority of cells that grew out from islets were nestin positive and insulin negative. B, NIPs that grew out from islets were picked, replated, expanded in the same medium (passage 1) for 3–5 d, fixed, and immunostained for nestin (*red*) and insulin (*green*). Most cells at this stage were nestin positive and insulin negative. C, Differentiation of NIP cultures treated with GLP-1. NIP cultures (clone 016f, passage 1) were expanded for 7–12 d. Between d 10 and 12, cultures were replenished with serum-free medium alone (control, C) or containing 10 nM GLP-1-(7–36)amide or GLP-1 plus exendin-(9–39) (100 nM; Ex9–39), a specific antagonist of GLP-1 (D and E, respectively). Seventy-two to 96 h later, cells were fixed and immunostained for nestin (Cy-3, *red*) and insulin (Cy-2, *green*). A subset of cells became nestin negative and insulin positive (indicated by *white arrows*, D). F, To control for background staining, GLP-1-treated cells were incubated with preimmune normal rabbit serum (*red*) and guinea pig IgG (*green*). Note the change in cell morphology when cells were treated with GLP-1 (D and F *vs.* C and E). G, Insulin secretion from NIP cultures treated with exendin-4. NIP cultures (passage 1) were expanded for 7–12 d and treated with 10 nM exendin-4, a GLP-1 agonist, for 48–72 h. Media were collected and assayed for insulin. Values are the mean ± SEM of four wells obtained from two different clones of NIPs.
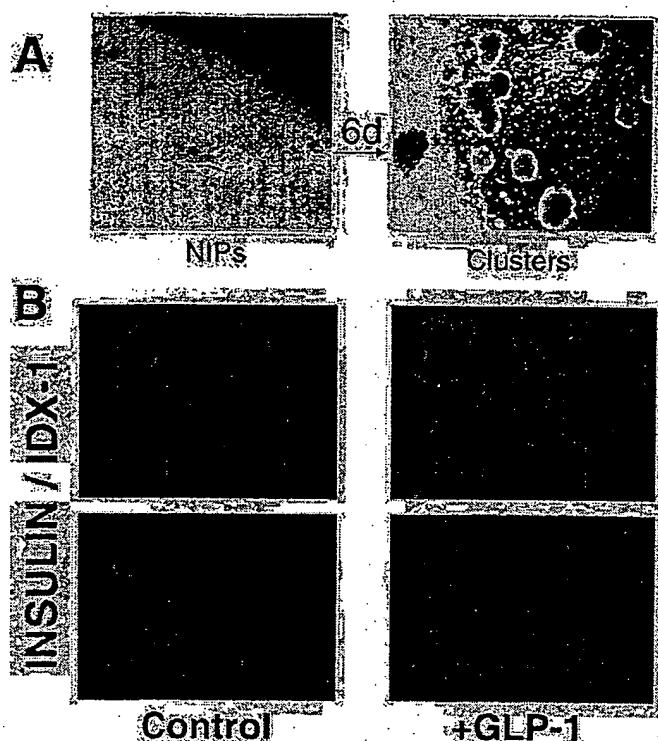
FIG. 4. Differentiation of long-term passaged (≤6 months) NIPs. A, Confluent NIP cultures (n = 2; clone 009b) were trypsinized and plated in B-27-supplemented medium (see *Materials and Methods*). The morphology of cells changed as they become more flattened (panel 1), and by 6 d the cells generated clusters (panel 2). B, The clusters were collected, centrifuged, and replated into laminin-coated 48-well plates in B-27 medium alone (control) or supplemented with 10 nM GLP-1. The cells that grew out from the clusters after 1 wk were then rinsed, fixed, and subjected to immunocytochemical detection of IDX-1 (Cy-3, *red*) and insulin (Cy-2, *green*).

A subset of cells became insulin and IDX-1 positive in the wells treated with GLP-1 as opposed to control cells without treatment with GLP-1 (Fig. 4B).

Transfection and expression of IDX-1 in NIPs

The homeodomain protein IDX-1 is critical for pancreas development (27) and plays a major role in transcriptional regulation of the insulin gene (15). It has been shown that GLP-1 agonists induce the expression of IDX-1 (8, 9) and that the expression of IDX-1 is sufficient by itself to induce the expression of insulin in liver cells (28) and in pancreatic ductal cells (12). We previously reported that IDX-1 is expressed in differentiated early passage NIP cell populations (14). We reconfirmed this observation in the current study using DNA binding assays with nuclear extracts prepared from differentiated confluent NIP cultures. A radioactively labeled, synthetic oligonucleotide probe encompassing the cytosine thymidine-II region of the human insulin promoter sequence formed a distinct complex that was eliminated when extracts were incubated with an antiserum for IDX-1, confirming the authenticity of IDX-1 in these cultures (data not shown). However, in long-term NIP cultures there was a loss/diminution of endogenous IDX-1 levels.

Next, we addressed whether GLP-1-induced differentiation of NIPs into insulin-expressing cells might correlate with the expression of IDX-1. Accordingly, we transiently transfected rat IDX-1 cDNA with a fragment of the rat insulin I promoter sequence conjugated to a luciferase construct (INS-LUC) into long-term (>6 months to 1 yr) NIP cultures and treated them with GLP-1 or forskolin. As shown in Fig. 5, reexpression of IDX-1 increased basal insulin promoter activity, and this effect was more pronounced when transfected NIPs were treated with GLP-1. In contrast, forskolin enhanced INS-LUC activity regardless of IDX-1 levels, suggesting that the GLP-1 effect on insulin gene expression in NIP cultures may be mediated by increased expression of IDX-1.

We also hypothesized that a certain concentration of IDX-1 in the cells in conjunction with the presence of GLP-1 is required for NIPs to convert into insulin-producing cells. To test this hypothesis, we treated NIPs transfected with human IDX-1 cDNA or untransfected long-term subconfluent NIP cultures (>3 months) with either 10 nM GLP-1 or vehicle for 3–4 d. Later, cultures were either fixed and immunostained with an antibody against human IDX (*red*) and insulin (*green*) or subjected to Western immunoblot analysis for nuclear IDX-1 protein. Immunostaining results show an overall increase in IDX-1 expression levels in transfected cells (four of five times) compared with untransfected cells (Fig. 6, A *vs.* B and C, *upper panels*). These findings were further confirmed by Western immunoblot analyses, which also suggest that GLP-1 treatment increases IDX-1 levels in NIPs transfected with an IDX-1 expression plasmid (Fig. 6D). However, insulin was induced in a subset of transfected and treated NIPs in only two of the above experiments, as shown in a representative experiment in Fig. 6C (*lower panel*) and was not
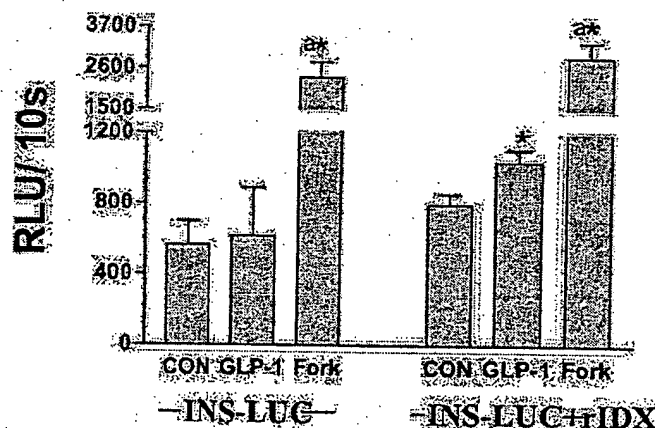

FIG. 5. GLP-1-induced differentiation of NIPs is mediated through a pancreas-specific transcription factor, IDX-1. A, NIP cultures transfected with −410 INS-LUC (INS-LUC) alone (*left*) or also with rat IDX-1 cDNA (*right*) were treated with forskolin (10 μM; Fork), GLP-1 (10 nM), or vehicle (CON) for 20 h. Then cells were lysed and assayed for luciferase activity. Relative light units (RLU) were measured for 10 sec (10s)/sample. Values represent the mean ± SEM of at least four wells from two experiments using clone 06. GLP-1-induced (*, *P* < 0.05) stimulation of INS-LUC activity is IDX-1 dependent, whereas that produced by forskolin (a*) is IDX-1 independent. Note that transfection with empty vectors (pxp2 and cytomegalovirus 5 promoter) yielded background units that were not altered by either treatment (data not shown).
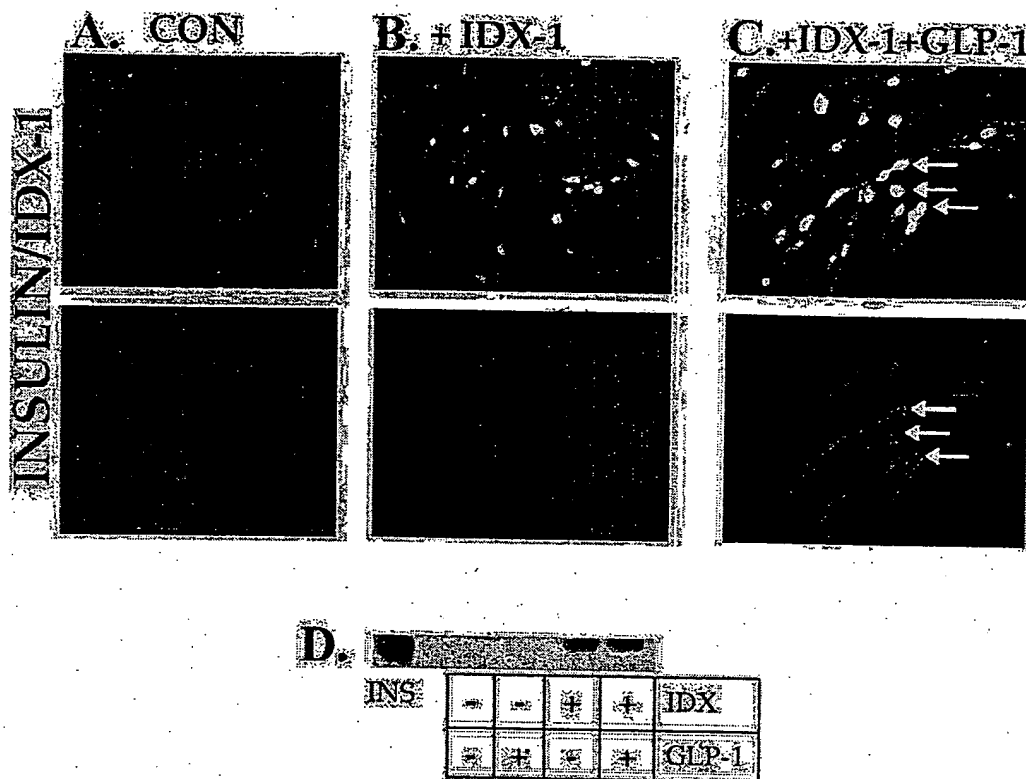
FIG. 6. A–C, Immunocytochemical analysis of insulin expression in NIP cultures transfected with human IDX-1. NIP cultures (passages 6–11) plated on Lab-Tek chamber slides were left untransfected (control (CON; A) or were transfected with human IDX-1 expression plasmid (+IDX-1; B and C). Five hours later, cultures were re-fed with medium containing serum. The following day, NIPs were treated with 10 nM GLP-1 (+IDX-1+GLP-1; C) or vehicle control (B). Three days later, cultures were fixed, permeabilized with methanol/Triton-X, and subjected to dual fluorescence immunocytochemical detection of IDX-1 (Cy-3, *red*) and insulin (Cy-2, *green*). Shown are representative figures in which NIPS (clone 006a) transfected with IDX-1 remains insulin negative/low (B, *lower panel*), and a subset of those treated with GLP-1 stains for insulin (C, *lower panel*). Note that IDX-1 immunostaining is more intense in those cultures that were transfected and treated with GLP-1 than in untransfected, untreated controls (B and C *vs.* A, *upper panels*). D, Western immunoblot analysis of IDX-1 expression levels in response to GLP-1. NIP cultures transfected with human IDX-1 cDNA or left untransfected were treated with GLP-1 (10 nM) or vehicle. Then cells were lysed, and nuclear extracts were prepared. Samples were electrophoresed and immunoblotted with an antibody specific for IDX-1. An extract from a rat insulinoma cell line (INS) was used as a positive control. Note the absence of endogenous IDX-1 in this clone of NIPs (>6 months in culture).

induced in other instances even in the presence of increased IDX-1 expression (Fig. 6B, *lower panel*). Taken together, these experiments suggest that IDX-1 may play a role in GLP-1-induced differentiation of NIPs into insulin-producing cells.

NIPs express the proglucagon gene and secrete GLP-1

Major regulators of expression of the nestin gene in neural stem cells are the POU homeodomain proteins Brn-2 and Brn-4 (29). Brn-4 is known to be a key activator of the expression of the proglucagon gene by interactions with the G_1 element located in the proximal α-cell-specific expression promoter of the proglucagon gene (30). Therefore, we examined NIPs for expression of the proglucagon gene. Although the proglucagon gene is not expressed in nonconfluent NIPs (Zulewski, H., unpublished observations), when NIPs approach confluence and begin to differentiate, they express the proglucagon gene, as shown by RT-PCR (Fig. 7A) and immunocytochemistry (Fig. 7B), and secrete GLP-1 into the culture medium (Table 1). Because NIPs express functional GLP-1Rs, the later expression of GLP-1 by early differentiating NIPs suggests that GLP-1 may function as an

autocrine /paracrine morphogen in the differentiation of multipotential NIPs to pancreatic endocrine cells.

Discussion

Our findings demonstrate the presence of functional GLP-1Rs on pancreatic progenitor cells and suggest a direct role for GLP-1 in the differentiation of NIPs into insulin-producing cells. Differentiation occurs in a subset of NIP cells that is induced to produce insulin. GLP-1 exerts diverse effects on β-cells, including stimulation of cAMP formation (25, 31) and activation of phosphoinositol 3-kinase (32–34), which, in turn, activates several downstream signaling targets that stimulate insulin secretion. We characterized the electrophysiological responses of NIPs to GLP-1 and found that NIPs bathed in physiological concentrations of glucose (5.6 mM) show an increase in $[Ca^{2+}]_i$ in response to the application of 10 nM GLP-1. Paradoxically, a higher glucose concentration (20 mM) rendered GLP-1 ineffective in evoking a $[Ca^{2+}]_i$ response. Like GLP-1, the cAMP agonist forskolin also evoked a similar increase in $[Ca^{2+}]_i$ in NIPs at 5.6 mM glucose, and the response to forskolin was abrogated at 20 mM glucose. These results suggest that NIPs
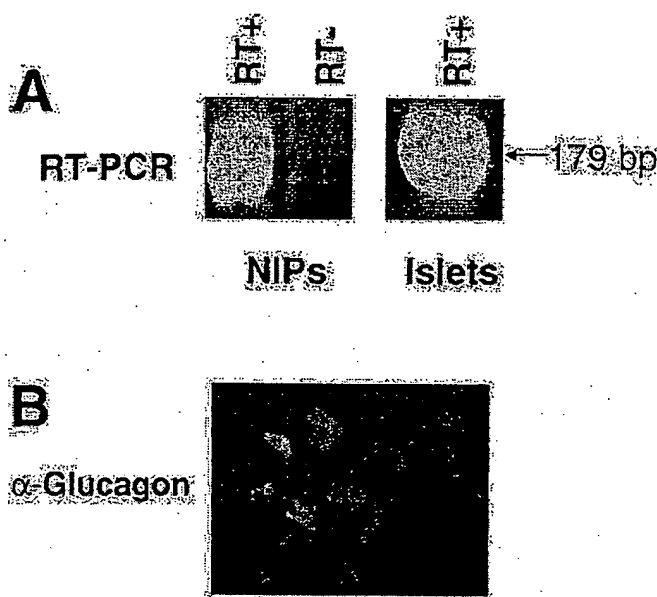
FIG. 7. A, Proglucagon is expressed in confluent NIP cultures. RT-PCR was performed of RNA prepared from NIPs (clone 9, passage 4) using oligonucleotide amplimers to human proglucagon giving the predicted 179-bp product. Islets were used as the positive control. B, NIPs were differentiated by culturing them in B-27-supplemented medium and plating them on wells (as described in *Materials and Methods*). Then cells were rinsed, fixed, and stained with an antiserum to glucagon (α-glucagon; Cy-2; intense *white* on modified figure).

TABLE 1. Secretion of GLP-1 by cultured NIPs

Well no.	GLP-1 (pg/ml)
1	12.7
2	56.7
3	79.8
4	21.2
5	48.1

Medium was collected from NIP cultures (passage 1), RIA was performed to assayed for secreted forms of GLP-1. The GLP-1 RIA is specific for the detection of the processed GLP-1-(7–36) amide and does not detect proglucagon, glucagon, or GLP-2. Shown are the values from five representative wells (clone 11).

contain a glucose-sensing mechanism. In support of these observations, our previous study demonstrates that Glut-2 mRNA is expressed in NIPs (14). Perhaps GLP-1 receptors are more abundant in conditions of physiological glucose concentrations, but are decreased in high glucose concentrations (35). Indeed, a recent study by Hui *et al.* (12) demonstrates that GLP-1R mRNA in ARIP cells (a pancreatic ductal cell line) is decreased in high glucose concentrations. Similar to islet β-cells, the NIP pancreatic progenitor cells respond to tolbutamide, a drug that binds sulfonylurea receptors on β-cells to depolarize them by closing ATP-sensitive K channels and to stimulate insulin secretion (36, 37). These studies demonstrate that functional sulfonylurea receptors are present on NIP pancreatic progenitor cells. Although our studies show the existence of tolbutamide-responsive ATP-sensitive K⁺ channels on NIPs and a depolarizing response to GLP-1, the apparent loss of augmentation of the response in conditions of high glucose (20 mM) is in contrast to the glucose-responsive sensitivity of β-cells to GLP-1 (25, 26). These observations suggest that unlike β-cells that increase their responsiveness to GLP-1 in conditions of acute elevations of glucose concentrations, the response of progenitor cells such as NIPs appears to be impaired in high glucose. It is important to distinguish between long-term glucotoxicity of β-cells in which cellular function is impaired after several days of exposure to high glucose (21, 38) and the acute impairment of NIP responses observed in just a few minutes. This apparent circumstance of the impairment of NIPs to respond to elevated glucose may be relevant to the deleterious effects of elevated blood glucose levels in individuals with diabetes, such that elevated glucose might impair the neogenesis of new β-cells from progenitor

precursor cells. Such glucotoxicity-mediated impairment of β-cell neogenesis, is expected to be accompanied by accelerated glucotoxic β-cell apoptosis (39–41).

The endocrine cells of the rat pancreas turn over every 40–50 d by processes of apoptosis and neogenesis (42). Neogenesis refers to the differentiation of new islet cells from progenitor cells residing in islets (14) and ducts (43–45). There have been several reports of the differentiation of pancreatic duct-derived cell lines into insulin-producing cells by growth factors (46), and GLP-1 is also implicated as a differentiation-inducing agent (10, 12). The administration of exendin-4 to rats stimulates β-cell neogenesis, resulting in increased β-cell mass (9). GLP-1 is now being considered as a potential new therapeutic agent for type 2 diabetic patients (13).

In their undifferentiated state, NIPs are nestin positive and IDX-1 and insulin negative. When exposed to GLP-1, a subset of cells became nestin negative and IDX-1 and insulin positive. Accordingly, insulin secretion by RIA was also detected in these cells. The differentiation of serially passaged NIP cultures into insulin-producing cells was accelerated by transfecting IDX-1 into NIPs before treating them with GLP-1. These findings are in agreement with those of Hui *et al.* (12), who also showed that transfection of PANC-1, a ductal cell line with IDX-1, followed by treatment with GLP-1 induced insulin biosynthesis.

The level of IDX-1 in NIPs may be critical; perhaps when NIP cultures are sequentially passaged, the level of endogenous IDX-1 falls, which is then corrected by transfecting in IDX-1. However, transfection with IDX-1 *per se* did not differentiate NIPs into insulin-producing cells. Treatment of IDX-1-transfected cells with GLP-1 was necessary to induce insulin biosynthesis in a subset of NIPs. Perhaps, transfection of IDX-1 into NIPs up-regulates GLP-1R expression, thus making it more responsive to its ligand. This was demonstrated by Hui *et al.* (12) using IDX-1 transfected PANC-1 cells. Consistent with our findings Wang *et al.* (47) demonstrated that the level of IDX-1 expression defines endocrine pancreatic gene expression. Like embryonic stem cells, the clonal variation in NIPs makes these kind of studies challenging in that responses to GLP-1 will depend not only on the presence or absence of its receptor, but also on the level of receptor expression (see Table 2 for frequencies of events).

Proglucagon gene expression appears to be restricted to endocrine pancreas, intestine, and brain (4, 5). The posttranslational processing of proglucagon is different in each of these tissues. Although the major proglucagon-derived peptide hormone in the pancreas is glucagon, GLP-1 is also

TABLE 2. Events/observations

Events/observations	Frequency	Clones tested
1. GLP-1 receptors on NIPs	≤60% (clone 006a)	3/3 clones; 006a, 009b, 002c; some have higher receptor expression than others
2. GLP-1 mediated differentiation of NIPS into insulin-producing cells		
a) Early passage	≤5–30%	3/3 clones; 010d, 011e, 016f
b) Long-term cultures		
Transfecting rIDX4 INS-LUC	≤66%	2/3 clones; 006a, 015g, 005h
Transfecting hIDX and immunostaining for insulin	↑ IDX-1 ≤80% ↑ Insulin ≤40%	3 clones tested; 006a, 013i,009b

produced. It is surprising that the proglucagon gene appears not to be expressed in nonconfluent passage NIPs, but then becomes expressed when the NIPs become confluent and differentiate. Brn-4 is known to activate nestin gene expression (29) and is a critical α-cell-specific activator of the proglucagon gene by interactions on the G_1 enhancer in the proximal promoter of the gene (30). Of note, we recently reported that the experimental misexpression of Brn-4 in the early developing pancreas (mouse embryonic d 8.5–9.5), directed by the IDX-1 promoter in transgenic mice, results in ectopic expression of the proglucagon gene in the later-developing insulin-producing β-cells (48). Because Brn-4 appears to play a critical role in the expression of both the nestin gene characteristic of stem cells (29) and the proglucagon gene, and the expression of Brn-4 at the time of activation of the IDX-1 promoter during development activates proglucagon gene expression (48), it is tempting to speculate that NIPs may be precursors of the IDX-1-expressing epithelial cells that appear in the foregut of the early mouse embryo (embryonic d 8.5–9.5) that is destined to give rise to the pancreas. Further, we speculate that the expression of the GLP-1R in NIPs coupled with the expression of proglucagon and resultant GLP-1 may establish an autocrine/paracrine hormonal feedback loop that is important in instructing the differentiation of NIPs into pancreatic endocrine cell lineages, *e.g.* β-cells. In addition, our findings are consistent with the reported observations that fetal pancreas-derived progenitor cells give rise to endocrine cells that initially express proglucagon (49) and later coexpress proglucagon and proinsulin (50).

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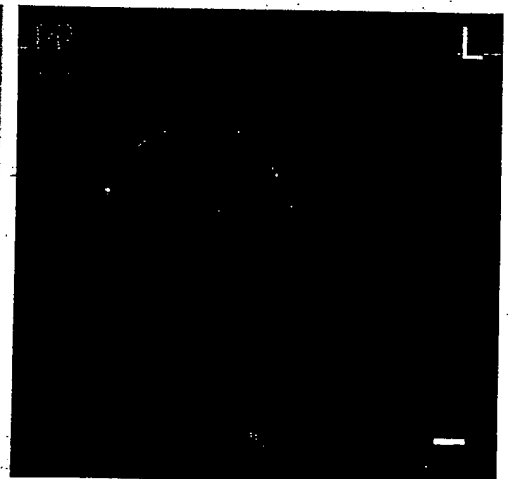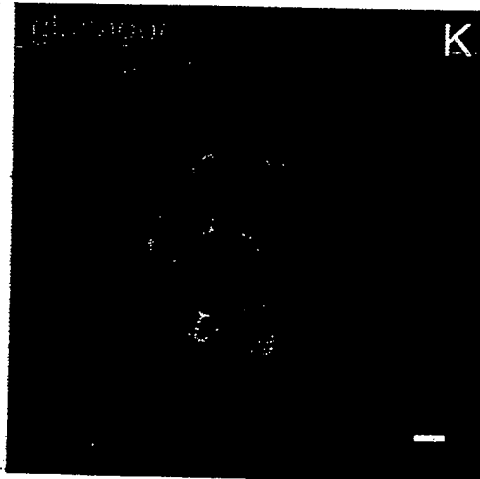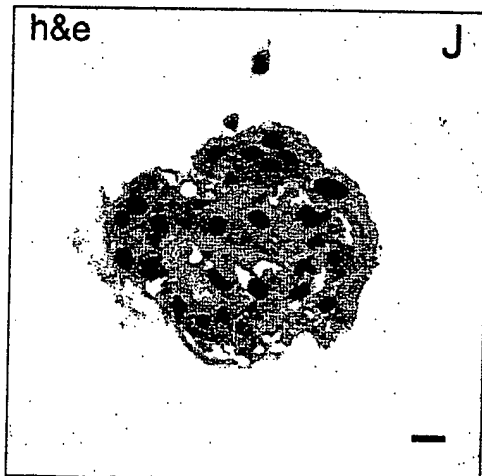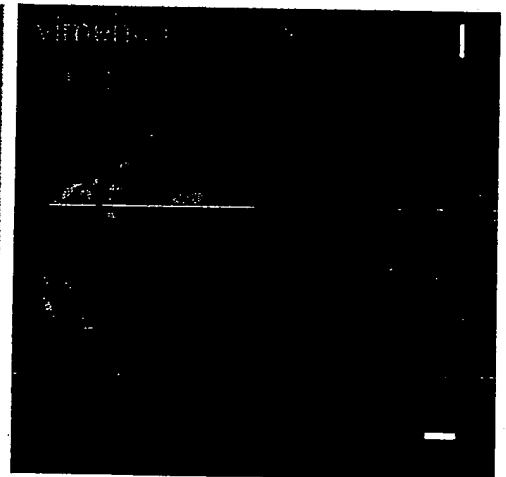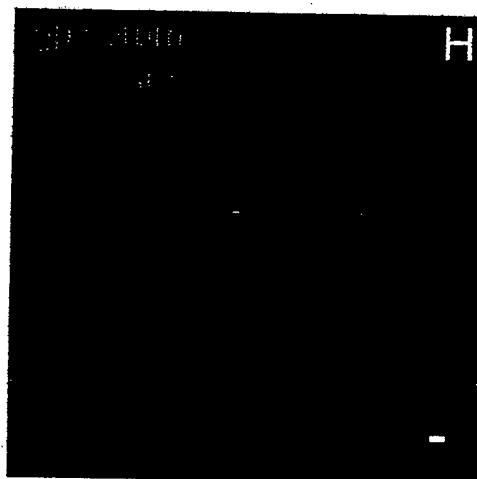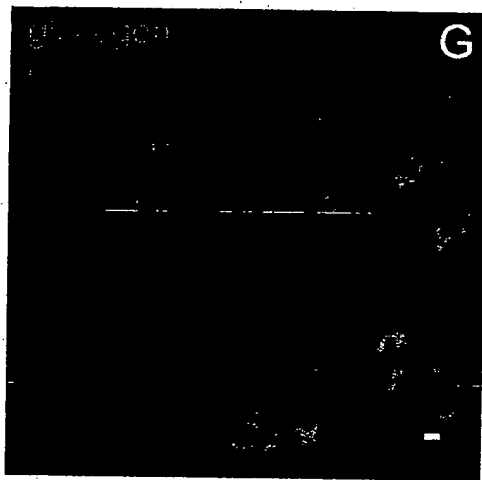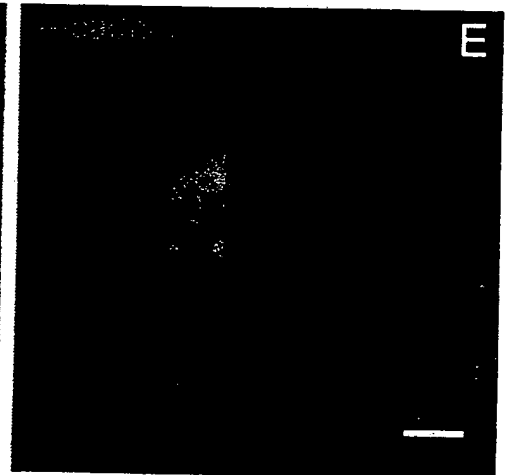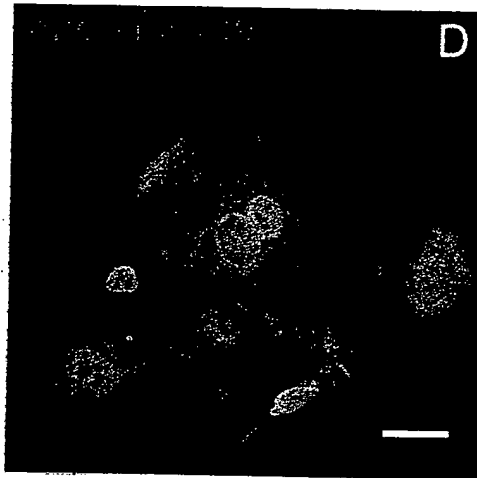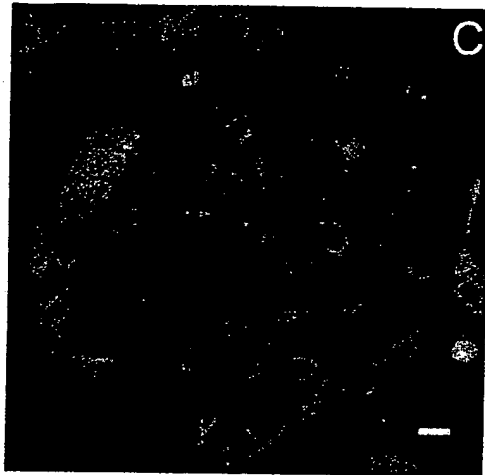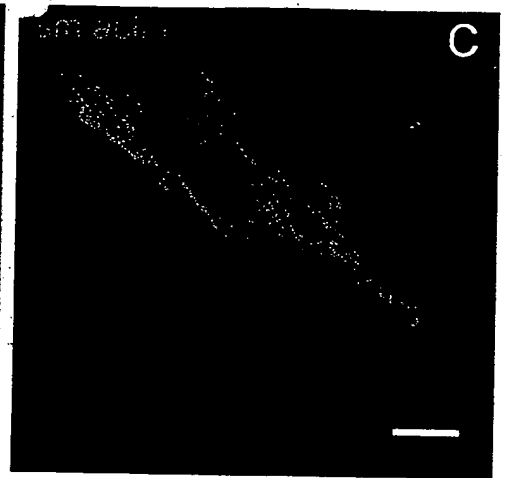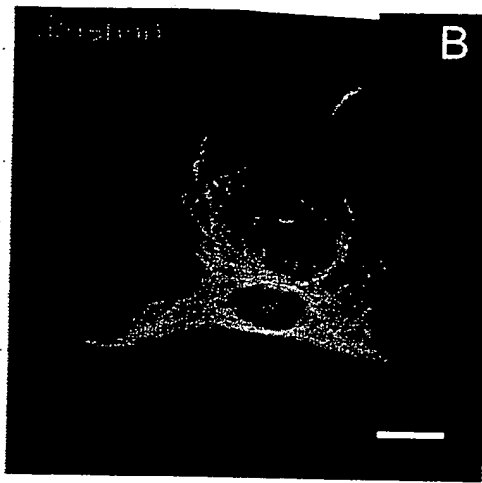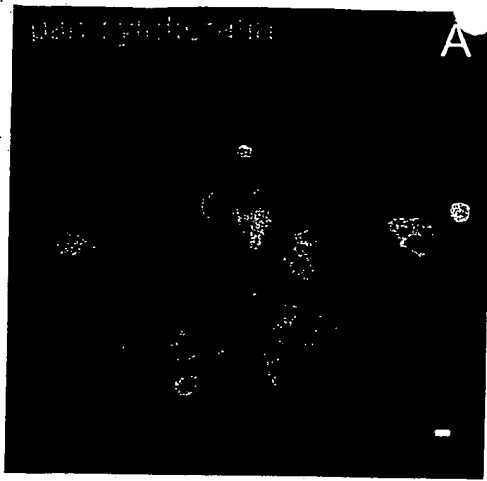
EXHIBIT C

CONFIDENTIAL

Michele Cimbala
December 21, 1998

Conception that Glucagon-Like Peptide-1 (GLP-1) Stimulates the Growth of

Pancreatic β-Cells

June 1986: I (Habener) met with Dr. Jeffrey Flier, a co-founder of a then forming new bio-technology company, Metabolic Biosystems, Inc. (MetaBio) to be a subsidiary of California Biotechnology, Inc. (Cal Bio). I discussed with him my ideas that GLP-1 appears to stimulate insulin secretion and may be a growth factor for pancreatic β-cells. Flier and I agreed that this was an exciting possibility. The parent patent on GLP-1 had been filed in May 1986. I was brought in as a consultant for MetaBio with the intention to examine the potential for GLP-1 to stimulate insulin secretion and the growth of new β-cells, and thereby to provide a potential treatment for individuals with diabetes mellitus, a disease known to be a consequence of an inadequate production of insulin by β-cells. We dis-cussed the existing information in the literature indicating that the inadequate production of insulin by the pancreas of diabetic individuals is due to a loss of function of β-cells and/or a reduction in the mass (numbers) of β-cells in the pancreas. Thus, if GLP-1 were to stimulate the growth and production of new β-cells, it would be a very promising po-tential therapy for the treatment of individuals with diabetes. This conceptualization of the potential for GLP-1 as a treatment of diabetes was clearly in my mind as of June 1986. The concept that the GRP encoded in the anglerfish preproglucagon discovered by us in 1981 and subsequently the orthologous peptide GLP-1 would/may stimulate the growth of β-cells in the pancreas was clearly established in 1981-1982. The whole idea that GLP-1 could be a treatment for individuals with diabetes, and would do so by stimulating the growth of new β-cells in the pancreas, was the major, motivating concept behind the deci-

sion of Cal Bio to establish a subsidiary company, MetaBio, to examine and develop GLP-1 as a therapeutic agent to stimulate insulin production, β-cell growth and functions, and hence β-cell mass in diabetic individuals. In June 1986 we had obtained initial data that GLP-1 stimulates insulin secretion but other hormones, such as glucose-dependent insulinotropic polypeptide (GIP) and cholecystokinin had already been shown to stimulate insulin secretion. Our interest was in whether GLP-1 could stimulate the growth of new β-cells in the pancreas.

7/87-5/ 88 Heather Hermann, a technician in my laboratory, and Habener conduct several studies in an attempt to directly demonstrate that GLP-1 stimulates the growth of pancreatic β-cells. GLP-1 and other proglucagon-derived peptides are added to cultures of insulinoma cells (β-cells) deprived of serum to arrest cell growth. The cells are pulse-labeled with ^{3}H-TdR and emulsion autoradiograms are prepared to examine rates of incorporation of ^{3}H into nuclei of β-cells, as an index of cellular proliferation. The experiments are technically difficult to interpret because the serum deprivation failed to arrest cell division. The concept that GLP-1 stimulates the growth of β-cells prevails. We conclude that the transformed insulinoma cells are not an adequate model to prove that GLP-1 stimulates β-cell growth.

1993-1994 We and other laboratories discover the transcription factor IDX-1 (PDX-1, IPF-1, STF-1) as a major regulator of insulin gene expression and the growth and development of the pancreas and the β-cells. The concept develops in my mind that maybe GLP-1 might stimulate the expression of IDX-1 and thereby may stimulate the neogenesis (new growth) of β-cells in the pancreas. I was then (and still am) excited by the idea that the expression of IDX-1 may be stimulated by cAMP signaling, similar to the transcription factor CREB, that we discovered in 1987. IDX-1, like CREB, had a cAMP-dependent phos-

phorylation site in the active region of the protein. Phosphorylation of CREB at its site is required for activity so I reasoned that IDX-1 activity may likewise depend on phosphorylation. That is, GLP-1 acts on its cellular receptors to stimulate cAMP signaling pathways that may activate the expression of IDX-1, which would then activate the differentiation and growth of β-cells. We recognized in 1985 that GLP-1 activated cAMP formation in β-cells (Drucker et al.). The GLP-1 receptor, cloned by B. Thorens, allowed direct confirmation that GLP-1 acts on a GPCR to stimulate the cAMP signaling pathway. The conception that GLP-1 would cause β-cells to differentiate and to grow was really firmly established and settled in my mind in 1993-1994 because of the discovery of IDX-1, which had cAMP-dependent phosphorylation sites in its structure (Lu et al. 1995). It seemed clear in my mind that GLP-1 indeed stimulates the neogenesis of β-cells.

June 1995   David Zangen presents a paper at the Eur. Acad. Soc. Diabetes describing our collaborative studies with the Joslin Diabetes Center showing that in the regenerating pancreas IDX-1 is highly expressed in the duct cells undergoing neogenesis to form new β-cells. The duct cells that first express IDX-1 go on to express insulin, indicating that the expression of IDX-1 correlates with the differentiation of ductal progenitor cells into the β-cells that produce insulin. The paper is submitted for publication to and sequentially rejected by *Development* and then by *PNAS*. The paper is now in press in *Diabetes*.

9/96-10/96   Habener orders customized matrix assisted delivery (MAD) GLP-1 pellets from Innovative Research of America, Inc. for implantation in to rats and mice to determine whether GLP-1 stimulates β-cell neogenesis, i.e. the differentiation and growth of new β-cells in the pancreas. I first called Innovative Research on 9/20/96 to discuss custom-made GLP-1 pellets. The experimental plan was to maintain GLP-1 pellet-implanted animals for three weeks and then inject the mice/rats with BrdU three hours before their sacrifice and to then examine the pancreata by *in situ* immunocytochemistry

with an antibody to BrdU to give an index of cell division rates. The pancreas sections were also co-stained with antiserum to insulin so that an increase of BrdU staining and insulin staining cells in the pancreatic ducts in response to GLP-1 would indicate that a stimulation of β-cell neogenesis had occurred.

These experiments were intended to provide preliminary studies to support my competing renewal application of my NIH grant DK30834 "Glucagon Biosynthesis and Metabolism."

1/97   The experiments were completed by Doris Stoffers, a postdoctoral fellow in my laboratory, who examined the sections of the pancreata from the mice treated with implanted GLP-1 pellets for 3 weeks with control mice implanted with dummy pellets. For technical reasons the staining with the antiserum to BrdU did not work. The concept that GLP-1 stimulates the neogenesis of β-cells still prevails.

2/28/97   The NIH grant application is submitted. It describes proposed studies with Josephine Egan at the NIA in Baltimore. A letter of intent to collaborate with Habener is provided by Egan and is appended to the grant application. The studies in the proposed collaboration are to determine whether GLP-1 will promote the neogenesis of β-cells in the pancreas.

6/96   At the Endocrine Society Meetings (ICE) held in conjunction with the ADA meetings, Habener introduces Stoffers to Egan and collaborative studies are discussed to determine whether GLP-1 may stimulate β-cell neogenesis and the growth of new β-cells in the pancreas. (There are some additional communications between Egan and me that I have to track down.)

3/97   Stoffers and Egan meet over lunch at the MEEI cafeteria to discuss concrete

plans of collaborative experiments to determine the effectiveness of GLP-1 to stimulate the

neogenesis of β-cells in mice. I was out of town and could not attend this meeting.

6/97   As agreed upon in the collaborative plan of 3/97 Egan brings the mice that

were infused with GLP-1 by subcutaneous osmopumps and mice that had been injected

daily with a long-acting GLP-1 agonist, exendin-4, at the NIA in Baltimore, to Boston the

week of the annual meeting of the American Diabetes Association, held in Boston, June

1997. On Saturday, June 21, 1997, the mice are sacrificed in my laboratory (Laboratory of

Molecular Endocrinology) at the MGH. The pancreata are obtained from the mice for

analyses by Western immunoblot and *in situ* immunocytochemistry (ICC) using our antis-

era to IDX-1, insulin, and other islet hormones.

7/97   Stoffers and I establish that GLP-1(7-36) and exendin-4 both stimulate the

expression of IDX-1 in the pancreas on Western immunoblots. Together we view images

of the ICC on the computer screen and mutually agree that it appears that the pancreata of

mice treated with GLP-1 agonists show an increased expression of IDX-1 and insulin in

the epithelial cells of the pancreatic ducts. We also quantitate the sizes of the islets in

GLP-1-treated vs. placebo-treated mice (done by a Summer Research Student Jeffrey Rhin

and show that the GLP-1 has increased islet mass by two-fold. This is exciting because

these observations indicate that GLP-1 has indeed stimulated β-cell neogenesis. The con-

cept that GLP-1 can stimulate β-cell neogenesis, the growth of new β-cells, is completed.

8-10/97   Mehboob Hussain, a postdoctoral fellow in my laboratory, treats

AR42J cells with the GLP-1 agonist exendin-4 and shows that the treatment (72 hrs) causes

an increase in the expression of IDX-1 and the expression of insulin in these cells. These

findings clearly indicate that GLP-1 can convert AR42J cells to β-cells that produce insulin. The background rationale for doing these experiments is that the AR42J cells were derived from a rat pancreatic carcinoma of ductal origin many years ago. The workers in the exocrine pancreas research have defined the AR42J cells as "amphicrine" cells because they have latent properties of both exocrine and endocrine pancreas cells. The addition of glucocorticoids, such as dexamethasone to AR42J cells converts them to an exocrine phenotype as the cells express amylase, chymotrypsin and other markers of exocrine pancreas cells in response to dexamethasone. Yet AR42J cells are electrically excitable, as are pancreatic endocrine cells. Then in 1995-96 several laboratories showed that treatment of AR42J cells with certain growth factors, such as betacellulin, TGFβ, activin A, hepatic growth factor could convert the cells to an endocrine phenotype that expresses insulin, like β-cells do.

We reasoned that if GLP-1 induces IDX-1 it (GLP-1) may also induce expression of insulin in AR42J cells in response to treatment with GLP-1 agonists. Thus the concept that GLP-1 stimulates pancreatic duct cells to turn on the expression of insulin, and thereby stimulates β-cell neogenesis, is completed again. The data obtained by Hussain are given in the preliminary results section of my resubmission of the amended NIH grant application DK30834 on 10/30/97.

6-7/97   I (Habener) call Egan and inform her of our promising and exciting results. Namely, it looks as though GLP-1 indeed stimulates β-cell neogenesis.

8/97   Egan comes to MGH and meets with Stoffers and me (Habener). We view the data together in the Wellman 3 conference room in the Laboratory of Molecular Endocrinology at the MGH. The computer images of the immunocytochemical staining of the pancreata from GLP-1-treated and saline placebo-treated mice are examined together.

Stoffers and I point out to Egan the evidence for neogenesis stimulated by GLP-1 treatment. Egan agrees that it certainly appears from these experiments that GLP-1 stimulates the neogenesis of β-cells because the enhanced expression of IDX-1 and insulin in the pancreatic ducts of the GLP-1-treated mice is much more pronounced than is seen in the ducts of the saline placebo-treated mice.

4/28/98   Egan gives a seminar at Endocrine Grand Rounds at the MGH. Egan shows data of the results of administration of GLP-1 agonists to mice and the treatment of AR42J cells with GLP-1 agonists (exendin-4). The data demonstrate that GLP-1 agonists stimulate the neogenesis of β-cells. This is of concern, because the data were presented by Egan as though done independently of us. The data were presented in a non-collaborative manner, as if all of the data originated from NIA without collaboration with MGH. It is also interesting that the reason Egan was invited to give Endocrine Grand Rounds is that Elahi asked me to invite her because she was up for promotion to a fulltime position at NIA, a promotion that requires evidence of independence in research, and that having been invited to give a seminar at MGH would look good on Egan's CV and may help obtain the promotion. So I had invited Egan to give this seminar sometime in July or August 1997.

Based on data presented by Egan at Endocrine Grand Rounds, Hussain indicates a loss of interest in continuing experiments of GLP-1 in AR42J cells that will only serve to duplicate Egan's experiments. We understand that the AR42J cell experiments presented by Egan at Endocrine Grand Rounds are in press in the *Journal of Clinical Investigation*.

Concept that the transcription factor IDX-1 (PDX-1/STF-1/IPF-1) is instrumental in the regulation of insulin expression and required for the development of the pancreas (β-cells)

Prior to 1994 it was known that GLP-1 stimulates the transcription of the insulin gene, enhances production of insulin in β-cells, and stimulates secretion of insulin, all in a glucose-dependent manner.

1993-1994 (dates depending on how long it took to get papers revised and accepted). The Laboratories of Habener, Montminy, and Edlund reported simultaneously that the homeodomain transcription factor IDX-1 is islet cell-specific and stimulates the expression of the insulin gene.

1994 Mice rendered nullizygous for *idx-1* are born without a pancreas--the pancreas fails to develop, pancreatic agenesis. Thus IDX-1 is required for not only regulation of transcriptional expression of the insulin gene but also for pancreas development. Stoffers joins my laboratory to do her research training. We learn about a child in Virginia born without a pancreas (pancreatic agenesis). Stoffers and I agree to examine the possibility that the child without a pancreas may be nullizygous for IDX-1. This turns out to be true. The child is homozygous for an inactivating mutation in the *idx-1* gene. We then learn that the child belongs to a very large extended family. The father and mother of the child are obviously hemizygous for IDX-1. The father was diagnosed as having diabetes at age 17. The mother also has diabetes. Examination of the extended family establishes that all carriers of the mutation in the *idx-1* gene have diabetes. Thus, haploinsufficiency in IDX-1 causes diabetes, and the diabetes is due to a lack of insulin production and secretion.

Therefore Habener reasons that if insufficiency in IDX-1 expression causes diabetes, and absence of IDX-1 arrests pancreas development, and that it is known that IDX-1 ex-

pression is restricted to β-cells of the adult pancreas that produce insulin, then IDX-1 may be important in the development of β-cells in the adult pancreas.

Further, and key to the conceptualization, is that GLP-1 stimulates insulin gene expression. Therefore GLP-1 may stimulate IDX-1 expression, and IDX-1 expression so stimulated may stimulate insulin expression. This is an important concept *because* the process of β-cell neogenesis, that is the formation of new β-cells by their differentiation from pleuripotential, or precursor cells in the pancreatic ducts, is believed (by me at least) to recapitulate the ontological development of the pancreas. It is well known that during embryonic development the pancreas is derived by the differentiation of gut endodermal epithelial cells that become the ducts of the pancreas and then give rise to the exocrine and endocrine pancreas (Islets of Langerhans).

1997   It is shown that mice hemizygous for IDX-1 get diabetes at 4-6 months of age. Also CreLox conditional knockouts of the *idx-1* gene get diabetes at 3-6 month of age. Thus in humans (discovered by us) and in mice, loss of *idx-1* expression causes diabetes. The diabetes is due to a reduction in β-cell mass and insulin production. GLP-1 is known to stimulate insulin production.

Importantly, it is well known (early 1990s) that GLP-1 binds to receptors on β-cells and stimulates the formation of cAMP. GLP-1 increases the levels of cAMP in these cells.

1997-1998   What controls the pancreas to develop from a small defined segment of the gut tube during early embryonic development? It was known that the pancreas develops as an evagination of the gut tube at e9.5 from an area of specialized prepatterned endo-dermal epithelium. Now we know that this specialized region of the gut tube must ex-press IDX-1 and also must not express the important developmental signaling molecule

Sonic Hedgehog (SHH). Given these two circumstances in the gut tube, expression of IDX-1 and repression of SHH, a pancreas will form.

Importantly, it is known that cAMP signaling is a potent antagonist to SHH signaling.

Therefore, I believe that GLP-1, by acting on receptors in pancreatic duct cells and generating cAMP in the adult pancreas, suppresses SHH, and activates IDX-1 expression, and thereby is the mechanism by which GLP-1 stimulates the differentiation of duct cells into β-cells, so-called neogenesis of β-cells. This process of neogenesis of β-cells in the adult pancreas is a recapitulation, a replay, of the embryonic development of the pancreas and of β-cells.

For consideration is my Summary

The concept that GLP-1 stimulates the growth of new β-cells was established in 1986 when it was discussed with Flier as a component for development by the newly formed company, Metabolic BioSystems, Inc. (Meta Bio). There should be letters, records at Scios to document this. Note that in 1986 the company was California Biotechnology, Inc. (Cal Bio) that then became Scios, then Scios Nova, then back to Scios again. Meta Bio was a subsidiary of Cal Bio.

The completion of the concept that GLP-1 stimulates the neogenesis of pancreatic β-cells probably occurred in 7/97.

EXHIBIT D

this a Clinical Trial? [ ]Yes [ ]No   Will it take place in Patient Areas? [ ]Yes [ ]No

Committee on Research (COR) Research Proposal Coversheet   (Instructions are on the back)

Joel F. Habener, M.D. _____   Chief, Laboratory of
NCIPAL INVESTIGATOR (Name, Degree/s Held)   Molecular Endocrinology
                                               MGH Title

ledicine/Molec. Endocrinol.   Wellman 320   6-5190
ARTMENT/SERVICE and UNIT   ADDRESS (Building/Floor/Room)   Telephone #

GLUCAGON BIOSYNTHESIS & METABOLSIM _____
ject Title

NCY __NIH NIDDK_____   Agency Application # R01 DK30834

ncy
dline __March 1, 1997__   Agency Type: [x]Government [ ]Foundation [ ]Industry

ase Indicate: [ ]New [x]Competing Continuation [ ]Non-competing Continuation [ ]Supplement

this a RESUBMISSION of A New or Competing Application? [ ]Yes [x]No

'E: [x]Grant   [ ]FIRST   [ ]RCDA   [ ]Other Type
     [ ]Contract   [ ]CIA   [ ]Training Grant
     [ ]Subcontract   [ ]CIDA   [ ]Fellowship

ase check here if the attached is an industrial contract [ ]

'LICANT ORGANIZATION: [x]MGH   [ ]HMS   Other Institution _____

:es: __01/01/98_____ to _12/31/98____   __01/01/98____ to _12/31/02___
         This 12-month Period   Entire project period

'AL DIRECT COSTS this 12-month period $ _138,136____   INDIRECT COST Rate _71%_

:ASE INDICATE:

nan Study(s)   [ ]Yes [ ]No   SHS#/ETC _93-4294_
ug Study(s)   [ ]Yes [ ]No
imal Study(s)   [x]Yes [ ]No
liation/Isotope Use   [x]Yes [ ]No   RAD/IS Approval Date _3/31/97_
ohazard(s)   [ ]Yes [ ]No
combinant DNA   [ ]Yes [ ]No
e of MGH NMR Facility   [ ]Yes [ ]No
e of MGH Cyclotron Facility [ ]Yes [ ]No

QUIRED SIGNATURES:
incipal Investigator _[signature]_____   Date _2/14/97_

ief of Department/Service _____   Date _____

DITIONAL SIGNATURE:
G. Chief of Staff,   Date _____
riners/Unit Chief, Etc.

PHS Certification Requirements - Conflicts of Interest

As of October 1, 1995, the NIH and NSF require all Principal Investigators, Co-Investigators, and all others responsible for the design, conduct, or reporting of the research (herein referred to as "Investigators") to disclose potential conflicts of interest between personal or family financial involvements and the research proposed in the grant application as submitted. These new requirements are detailed in 42CFR part 50, which is available in the Research Administration office.

To summarize the requirements, each Investigator identified in a grant application must disclose to the Institution any "Significant Financial Interests" (i) that would reasonably appear to be affected by the research for which the funding is sought, and (ii) in entities whose financial interests would reasonably appear to be affected by the research. "Significant Financial Interest" means any salary or other payment for services, royalties, and any other payments from a company that in the aggregate exceed $10,000 per year, and any equity interest that exceeds $10,000 or 5% ownership in an entity. The "Significant Financial Interest" includes the aggregate interests held by the Investigator and his/her spouse and dependent children as well. Any conflict of interest must be managed, reduced, or eliminated, as determined by the Institution in accordance with its policy, before a grant can be activated.

All Institution researchers now are required to disclose annually to the Institution and the Harvard Medical School all potential conflicts of interest as defined by the Institution and HMS. The new requirement for disclosure with respect to PHS funding of research now is a part of the Institution and HMS disclosure requirements. Therefore, such disclosures must be included during the annual reporting.

To make possible the submission of a grant application, the PI must insure that all Investigators proposed as participants in the grant disclose any Significant Financial Interest, or certify that they have no such Significant Financial Interest, when the grant application is submitted to the Administration for institutional approval. This form is designed to facilitate such disclosure.

Investigator Name: _____ Joel F. Habener, M.D. _____

Title of Grant Application: _____ Glucagon Biosynthesis & Metabolism _____

I hereby certify that I (including my spouse and dependent children):

- ☒ have no Significant Financial Interest as defined by NIH/NSF policies

- ☐ have Significant Financial Interest as defined by NIH/NSF policies as follows:
  (n.b. - If you have not disclosed this Significant Financial Interest to Harvard Medical School with respect to your activities as a faculty member at that institution, you must complete the HMS form with this information and include that form in a separate, sealed envelope with this grant application.)

- Employment, Consultant, or Royalty: (income from which, in the aggregate [including that to my spouse and dependent children], exceeds $10,000 per year):
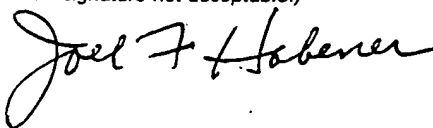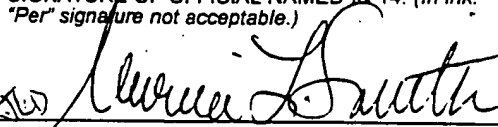
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_____

- Financial Interests, including Equity: (which exceeds $10,000 or 5% share of the entity [including that held by my spouse and dependent children]):

_____

_____

Signed - Investigator   February 27, 1997
                                           Date   (RA-9/95)

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK—FOR PHS USE ONLY.

Type	Activity	Number
Review Group	Formerly	
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
GLUCAGON BIOSYNTHESIS & METABOLISM

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT [x] NO [ ] YES *(If "Yes," state number and title)*
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*
Habener, Joel Francis

3b. DEGREE(S)
M.D.

3c. SOCIAL SECURITY NO.
572-50-7801

3d. POSITION TITLE
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3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Medicine

3g. MAJOR SUBDIVISION
Molecular Endocrinology

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4. HUMAN SUBJECTS
[x] No
[ ] Yes

4a. If "Yes," Exemption no. or
IRB approval date { [ ] Full IRB or [ ] Expedited Review

4b. Assurance of compliance no.
M1331-01

5. VERTEBRATE ANIMALS
[ ] No
[x] Yes

5a. If "Yes," IACUC approval date
2/19/97

5b. Animal welfare assurance no.
A3596-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*
From 01/01/98 | Through 12/31/02

7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD
7a. Direct Costs ($) 138,136 | 7b. Total Costs ($) 228,340

8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT
8a. Direct Costs ($) 748,192 | 8b. Total Costs ($) 1,236,769

9. APPLICANT ORGANIZATION
Name Massachusetts General Hospital
Address The General Hospital Corp.
55 Fruit Street
Boston, MA 02114

10. TYPE OF ORGANIZATION
Public: → [ ] Federal [ ] State [ ] Local
Private: → [x] Private Nonprofit
Forprofit:→ [ ] General [ ] Small Business

11. ORGANIZATIONAL COMPONENT CODE 30

12. ENTITY IDENTIFICATION NUMBER
1042697983A1

Congressional District 9

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name Marcia L. Smith
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14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
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E-Mail Address smith@helix.mgh.harvard.edu

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI / PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*
[signature: Joel F. Habener]

DATE 2/27/97

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. *(In ink. "Per" signature not acceptable.)*
[signature: Marcia L. Smith]

DATE 2/27/97

PHS 398 (Rev. 5/95)   Face Page   AA

GLOSSARY of Abbreviations

Brn-4   pou-specific homeodomain protein Brain-4

CCK   Cholecystokinin

CHOP   c/EBP homologous protein

CRE   cAMP-response element

CREB   Cyclic AMP response element binding protein

CRH   Corticotropin-releasing hormone

GHRH   Growth hormone-releasing hormone

GIP   Gastric inhibitory peptide

GLP-1   Glucagon-like peptide-1

GLP-1R   Glucagon-like peptide-1 receptor

IDX-1   Islet duodenal homeobox protein

K-ATP   ATP-sensitive K^+ channels

MPF   Major proglucagon fragment

MyoD   Muscle specific transcription factor

NIDDM   Non insulin dependent diabetes mellitus

PACAP   pituitary adenylyl cyclase-activating protein

Pan-1   Transcription factor E47

PTH   Parathyroid hormone

SUR   Sulfonylurea receptor

VDCC   Voltage-dependent Ca^{2+} channels

VIP   Vasoactive intestinal peptide

DESCRIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concis: the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is me: to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this descrip: : as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The disease non insulin dependent mellitus (NIDDM) is increasing by epidemic proportions in developed countries throughout the world. There are an estimated 100 million individuals with diabetes and an equal number who have not yet been diagnosed. One important manifestation of NIDDM is impaired and/or dysregulated secretion of the hormones insulin and glucagon in conjunction with impaired insulin sensitivity (insulin resistance). Glucagon is a catabolic hormone whose physiological actions are counter to those of the anabolic hormone, insulin. Hyperglucagonemia is a common manifestation of diabetes, increases hepatic glucose output, and worsens hyperglycemia. The overall hypothesis being tested in these studies is that the regulation of the expression of the glucagon gene is critically important during the switch from fasting (catabolic) to the fed (anabolic) state. The glucagon gene is expressed in both the pancreas and the intestine. Remarkably, by mechanisms of alternative post-translational processing of proglucagon, the pancreas produces the bioactive peptide glucagon, the anti-insulin hormone important in the fasting state to maintain blood glucose levels. In the intestine the bioactive hormone produced is glucagon-like peptide-I (GLP-I), an incretin hormone that has potent insulinotropic actions on β-cells of the pancreas, satiety actions on the hypothalamus, and possible peripheral actions on adipose and skeletal muscle to enhance glucose uptake and on liver to inhibit glucose output. It is proposed that: 1) During fasting, glucagon gene expression is tonically elevated due to the low insulin and glucose levels and high neuroadrenergic inputs likely mediated by cAMP-dependent signaling pathways. 2) During feeding, oral nutrients induce intestinal L-cells to release the insulinotropic hormone GLP-I that activates specific cAMP-coupled receptors on pancreatic β-cells and, synergetically with glucose, stimulates insulin and represses glucagon release and production, respectively. We propose to continue our investigations of the mechanisms involved in the transcriptional expression of the glucagon gene. The aims are to: (1) examine the potential role of the pou-specific homeodomain protein Brain-4 in the α-cell-specific expression of the proglucagon gene and as a possible factor in α-cell development; (2) isolate, identify, and characterize the peripheral GLP-1 receptor expressed on adipocytes. We propose to clone the receptor from a 3T3-L1 cell cDNA expression library, prepare stable cell lines expressing the receptor, characterize the hierarchy of peptide hormone binding and the coupling to signal transduction pathways, and investigate the potential role of the receptor in diabetes; (3) examine the potential properties of GLP-1 to enhance growth and to inhibit apoptosis of pancreatic β-cells. The importance of hormones encoded by the glucagon gene in the maintenance of glucose homeostasis, and their potential relevance to the pathogenesis of NIDDM, provides interest in learning more about the controlling factors involved in the expression of the gene.

PERFORMANCE SITE(S) *(organization, city, state)*

Massachusetts General Hospital

Boston, MA

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Joel F. Habener, M.D.	Massachusetts General Hospital	P.I.
Colin A. Leech, Ph.D.	Massachusetts General Hospital	Research Assoc
Karen S. McManus	Massachusetts General Hospital	Technician

Type the name of the principal investigator/program director at the top of each printed page and each continuation page. (For type specifications, see instructions on page 6.)

RESEARCH GRANT

TABLE OF CONTENTS

Research Plan

*Type density and type size of the entire application must conform to limits provided in instructions on page 6.

Appendix (*Five collated sets. No page numbering necessary for Appendix.*)

     Number of publications and manuscripts accepted or submitted for publication (*not to exceed 10*)   10

     Other items (*list*):

[X] Check if Appendix is included

the peripheral GLP-1R no longer generates cAMP, but rather acts through Ca²⁺ or PI or other signaling pathways to stimulate lipogenesis and not lipolysis.

Several lines of investigations of the novel GLP-1 receptor may be productive. One would be to ask whether this receptor is also expressed in the liver and muscle, or even brain or islet cells, and to determine the tissue distribution of the receptor by using approaches of RT-PCR of tissue extracts, RNase protection assays. *in situ* histohybridization, and immunocytochemistry and Western immunoblots, such as we did for the pancreatic-type GLP-1R described in our paper **P5**. One of the first things that we will do is to prepare an antiserum to the novel GLP-1R using a synthetic peptide to a sequence estimated to be just carboxy-proximal to the N-terminal signal sequence of the receptor.

It would be interesting to investigate the ion channel activities and effects on $[Ca^{2+}]_i$ of the ligand-activated receptor in single cells using the patch clamp electrophysiological approaches and fura-2 dual-wave length calcium imaging. Ion channel activities could be examined in the L-6 myocytes because they are electrically excitable cells.

If GLP-1 enhances insulin stimulated glucose uptake in cells (3T3-1 adipocytes) as reported by Egan et al. [55], it may do so by enhancing the translocation of glucose transporter-4 to the plasma membrane. This can be examined by measuring the change in capacitance of the cell in response to GLP-1.

Another potentially interesting line of investigation might be to determine whether the novel peripheral GLP-1R is upregulated or downregulated in animal models of diabetes. Circulating GLP-1 levels are reported elevated in diabetic rats, as well as in NIDDM subjects [62, 63]. Therefore, increased circulating GLP-1 may contribute to obesity in diabetic subjects. If the peripheral GLP-1R is coupled to signal transduction pathways different from the cAMP pathway to which the pancreatic GLP-1R is coupled it may not desensitize. One might speculate that eventual administration of GLP-1 to NIDDM subjects might enhance peripheral glucose utilization, in addition to stimulating insulin secretion and inhibiting feeding behavior.

Another line of investigation might be to examine the cross-talk between the leptin cytokine and the GLP-1 signaling pathways. Leptin is the obesity (starvation) hormone made in fat that inhibits feeding, increases energy expenditure and reproductive processes. Leptin also has receptors on adipocytes (recent Keystone meeting) and inhibits insulin-mediated glucose uptake in skeletal muscle (recent Keystone meetings on diabetes and obesity). Cyclic AMP signaling is well recognized to antagonize cytokine signaling. Since we have recently found that leptin inhibits insulin secretion by hyperpolarizing β-cells via opening ATP-sensitive K⁺ (K⁺-ATP) channels, and the leptin receptor (Ob-Rb long form) is coupled to the Jak/STAT and possibly MEKK/ERK cytokine pathways, GLP-1 signaling may act on K-ATP (Kir6.2 or SUR) or on Ob-Rb, or on Jak or STAT to antagonize leptin inhibition. Such a cross-talk between GLP-1 signaling and leptin signaling may explain how insulin can be secreted during meals when incretins such as GLP-1 are released and overcome the inhibitory actions of leptin on insulin secretion. Further avenues of investigation of the peripheral GLP-1R would be to examine the gene structure and determine whether alternative exon-splicing occurs and modifies receptor activity, examine the promoter and identify transacting factors that may be expressed during the differentiation of 3T3-L1 adipoblasts to adipocytes, thereby activating the transcription of the receptor gene. The physiologic importance of the GLP-1R can be tested by producing mice with a targeted disruption of the gene, as has been done for the pancreatic GLP-1R by Drucker et al., resulting in a phenotype of glucose intolerance. Double transgenic mice may be created by crossing mice with knockouts of the peripheral GLP-1R to mice with knockouts of the pancreatic GLP-1R.

3. Investigations of potential actions of glucagon-like peptide-1 (GLP-1) on β-cell differentiation, proliferation, and apoptosis

The hypothesis to be examined is that the long-term administration of GLP-1 may enhance β-cell mass. We plan to administer GLP-1 long-term to streptozotocin diabetic mouse and rat models and to directly test the effects of GLP-1 on β-cell proliferation, differentiation, and apoptosis and thereby to establish whether or not GLP-1 may be a determinant of β-cell mass. Three experimental approaches are proposed: (1) The aged diabetic Wister rat model (in collaboration with J. Egan, NIH Age Institute); (2) The regenerating pancreas model (in collaboration with Drs. Gordon Weir and Susan Bonner-Weir, Joslin Research Laboratories) and (3) The transgenic IDX-1 promoter-LacZ reporter mice that we have generated in our laboratory (Stoffers et al., submitted for publication). Before describing the details of the experimental approaches, some background and rationale for justifying the undertaking of these experiments is required.

The actions of GLP-1 on its receptor, at least in β-cells, generates high cellular levels of cAMP. Cyclic AMP is well known to stimulate the proliferation of many different cell types and to promote differentiation of other cell types [reviewed in 120]. Phosphorylated CREB also activates the transcription of the BCL-2 gene, increases cellular levels of BCL-2, and rescues apoptosis in B-lymphocytes [126]. BCL-2, the mammalian homologue of the nematode protein Ced-9 (c. elegans death protein-9), is a potent universal inhibitor of apoptosis [127, 128] (**Fig. 16**). BCL-2 is a 26 kDa protein, located in membranes of the mitochondria and endoplasmic reticulum, that is believed to protect against apoptosis by decreasing the net cellular generation of reactive oxygen species (ROS)

and lipid peroxidation. Notably, glycation end products are implicated in the generation of ROS and chronic hyperglycemia enhances the formation of glycation end-products. Furthermore, glycation-dependent ROS appear to mediate the suppression of insulin promoter activity in hamster insulinoma (HIT) cells [129, 130]. A number of BCL-2 homologs have been identified, including BCL-X$_L$, and BAG-1, and Bad, which promote apoptosis. Current evidence suggests that the ratios of these anti-to pro-apoptotic proteins may play a regulatory role in apoptosis [131].

It seems plausible, therefore, that long-term administration of GLP-1 to rat or mouse models of reduced β-cell mass and impaired β-cell function may either stimulate β-cell proliferation/differentiation and/or inhibit apoptosis (**Fig. 17**). If GLP-1 does have any of these actions, their demonstration would be relevant to the rational for the long-term treatment of diabetic subjects, not only those with NIDDM, but also possibly insulin-dependent diabetes mellitus (IDDM) type I, juvenile diabetes in which the β-cell mass is severely reduced but in which progenitor β-cells located in the pancreatic ducts remain viable. To date, few long-term studies of GLP-1 administration have been done, particularly studies in which the parameters of β-cell proliferation, differentiation, and apoptosis have been examined. As described in C. Progress Report/Preliminary Studies, 48-hr infusions of GLP-1 to aged diabetic (23 month old) rats results in a marked stimulation of insulin secretion and production and in β-cell proliferation (J. Egan, NIH Aging Institute, submitted for publication). We are planning to collaborate with Dr. Egan to examine the effects of the GLP-1 infusions on the expression of IDX-1 in the β-cells (see letter of intent to collaborate, Appendix). It appears that the transcription factor IDX-1 is a positive regulator of insulin gene expression and is involved in pancreatic development. That IDX-1 is involved in the differentiation of progenitor pancreatic duct cells into insulin-producing β-cells is supported by a collaborative study done with Dr. Gordon Weir at the Joslin Research laboratories (Zangen et al., submitted for publication) using the regenerating pancreas rat model following partial pancreatectomy. During the first days following 90% pancreatectomy, the pancreatic remnant undergoes an intense proliferative phase of the ductal cells followed by the expression of insulin and glucagon as the ductal cells differentiate into endocrine cells. In essence, this regenerating pancreas model recapitulates the ontological development of the pancreas. We have observed a marked increase in IDX-1 and insulin gene expression between days 2 and 3, corresponding to the transition from the proliferative to the differentiation phase of regeneration. These findings support the idea that IDX-1 is involved in the differentiation of ductal progenitor β-cells to mature β-cells. Further, these initial findings raise the possibility that the regenerating pancreas model may be a means to test the hypothesis that GLP-1 and cAMP signaling may stimulate the process of the differentiation of ductal progenitors of β-cells to mature insulin-producing cells.

Experimental methods
Long-term administration of GLP-1 to rats and mice
A proven effective method to deliver hormones to rats and mice is to use implanted mini osmopumps (Alzet. Inc.). However, the pumps do get plugged and fail to deliver the hormone, for technical reasons (our observations). As an alternative to osmopumps, we have been experimenting with matrix assisted delivery pellets, prepared by Innovative Research America, Inc. We provided them with 20 mg of GLP-1(7-37) which they formulated into time-release pellets for our use. The pellets are inserted under the skin of rats or mice with a trochar. The pellets deliver GLP-1 for up to 21 days and achieve blood levels of 20-50 pM equivalent to prandial levels. Placebo pellets are provided to serve as controls. However, we have not yet thoroughly evaluated matrix-assisted delivery pellets for delivery rates and pharmacodynamics. We do have preliminary data on osmopumps that are encouraging and they can be used if matrix pellets don't work. We tested both 0.2 ml and 2.0 ml 7-day delivery pumps implanted subcutaneously in the nape of 500 gm rats. The GLP-1(7-37) solutions contained 2 mg (0.2 ml) and 10 mg (2.0 ml). At the end of 5 days of GLP-1 administration, the animals were sacrificed. Plasma GLP-1 levels achieved were 4 ng/ml and 0.8 ng/ml for the 2.0 ml and 0.2 ml pumps, respectively (T1/2 3-4 min, MCR 11 to 14 ml.min). This rangefinder study allows us to calculate the desired dose of GLP for administration. Normal basal and prandial GLP-1 levels are 2 and 10 pM and 10 and 60 pM for the 7-37 and 7-36amide isopeptides, respectively. We would aim for infusion plasma levels of 50-100 pM (150-300 pg/ml). Thus, the smaller pumps (0.2 ml) containing 0.5-1.0 mg GLP-1(7-37) can be used.

Creation of streptozotocin diabetic mice and rats
Streptozotocin is a relatively specific β-cell toxin, believed to act by specifically compromising the NADH-ryanodine, cyclic ADP ribose metabolic system unique to β-cells. Streptozotocin can be administered at low doses to create "NIDDM" models and at higher doses to create "IDDM" models of diabetes. The protocols for generating these mouse and rat models of diabetes are well documented [132, 133].

Experimental parameters to be evaluated
The extent of impaired glucose tolerance (IGT) or diabetes in response to streptozotocin will be monitored by measuring urine and plasma glucose levels, and by oral glucose tolerance tests [134] in which we will measure blood glucose, insulin, and GLP-1 levels.

(i) The cAMP antagonist (Rp cAMPs) also evoked the current, suggesting that the cAMP actions were not mediated by PKA, but perhaps by the binding of cAMP to a protein, perhaps the Na/Ca-NS. (ii) The elicited current is voltage independent and not inhibited by VDCC blockers such as nifedipine and veraparmil or by clamping the voltage at -70 to -100 mV at which VDCCs cannot open. (iii) The activation of the current is also totally dependent on extracellular Na^+ and Ca^+ chelators BAPTA-AM or EGTA, suggesting the participation of a Na^+/Ca^+ exchanger and movement of Ca^{2+} from intracellular stores, respectively. **Figure 9** provides a model with which to evaluate these diverse actions of GLP-1. Receptor occupancy by GLP-1 activates $G_{s\alpha}$ proteins and stimulates adenylyl cyclase, thereby accelerating conversion of ATP to cAMP. We propose that this catalytic process is dependent on extracellular Na^+ and that the subsequent binding of cAMP to cyclic nucleotide-regulated non-selective cation channels (or a protein closely associated with the channel) results in channel activation, thereby generating I_{cAMP}. Activation of these channels by cAMP is also proposed to require intracellular Ca^{2+}. The rise of $[Ca^{2+}]_i$ which accompanies I_{cAMP} is achieved by stimulation of at least two distinct Ca^{2+} signaling pathways. First, the membrane depolarization that is a direct consequence of I_{cAMP} results in activation of VDCCs, thereby raising $[Ca^{2+}]_i$. Second, a rise of $[Ca^{2+}]_i$ is observed even under conditions in which the membrane potential is voltage-clamped at values (-100 to -70 mV) negative to the activation threshold of VDCCs. Although the nature of this additional rise of $[Ca^{2+}]_i$ remains to be determined, it may signify the mobilization of Ca^{2+} from intracellular stores, as well as Ca^{2+} influx via nonselective cation channels and/or membrane transporters (see below). Acting in concert, these Ca^{2+} signaling pathways are proposed to contribute to the stimulatory actions of GLP-1 on insulin secretion from β-cells.

From a functional standpoint, the ability of GLP-1 to raise $[Ca^{2+}]_i$ through activation of a signaling system and not involving effects on I_KATP has at least one important ramification. GLP-1 augments insulin secretion in non-insulin-dependent diabetics, even under conditions in which the sulfonylurea drugs such as glyburide (which inhibits I_KATP) fail to stimulate insulin secretion (sulfonylurea failure). This observation suggests that one therapeutic advantage of GLP-1 relative to that of sulfonylureas in the treatment of non-insulin-dependent diabetes is that GLP-1 triggers a rise of $[Ca^{2+}]_i$, insulin secretion, and a lowering of blood glucose, even under conditions in which sulfonylurea receptors and ATP-sensitive potassium channels no longer play a dominant role in the regulation of β-cell stimulus-secretion coupling. Therefore, activation of I_{cAMP} by GLP-1 may serve as a reserve mechanism of action, one that complements its previously reported inhibitory effects on I_KATP. This would then explain why the glucagon-like peptides retain their biological activity and augment insulin secretion even under conditions in which sulfonylureas are no longer effective.

We also showed that the voltage independent Na^+/Ca^{2+}-NS channels are involved in the slow oscillations in β-cells (**P8**) and are part of the β-cell depolarization mediated by pituitary adenylyl cyclase-activating protein (PACAP) (**P9**). The spontaneous slow oscillations in cytosolic calcium in β-cells are mediated by voltage-independent channels (**P8**). These observations suggest that the slow oscillations in $[Ca^{2+}]_i$ may serve as important initiators of insulin secretion under conditions in which tight control of glucose homeostasis is necessary such as during the fasting normoglycemic state. It is proposed that PACAP may be important in a neuro-entero-endocrine loop regulating insulin secretion during the transition period from fasting to feeding (**P9**).

Potential trophic effects of GLP-1 on β-cell neogenesis and proliferation

Several lines of evidence suggest the possibility that GLP-1 may have trophic actions on β-cells. GLP-1 is known to stimulate cAMP formation in β-cells and to stimulate insulin secretion, and also insulin biosynthesis. Further, it is known that cAMP is an effective second messenger and stimulates certain cell types to proliferate and in other cell types inhibits proliferation and induces cellular differentiation [120]. Essentially all of the studies of GLP-1 actions conducted thus far have been short term experiments to demonstrate the insulinotropic actions, i.e., to stimulate insulin secretion and to lower blood glucose levels. In preliminary studies with Dr. J. Egan of the Aging Institute 48 hr continuous infusions of GLP-1 have been administered by subcutaneously implanted mini osmopumps to aged rats (23 months old). This particular strain of Wister rats develops hyperinsulinemia and glucose intolerance at about one year and by 18 months develop diabetes akin to NIDDM. The results of initial studies of the 48 hr infusions of GLP-1 to aged rats has provided some provocative results. Insulin secretion and glucose tolerance (oral) in these aged rats, several hours after the termination of the GLP-1 infusion, was normalized to that of the younger 6 months old rats with normal insulin secretion and glucose tolerance. Further, by immunostaining sections of the pancreas and RIA of extracts of pancreas, insulin content increased by 2-fold compared to sham infused control rats. In effect, the 48 hr infusion of GLP-1 converted the glucose/insulin physiology of aged rats with impaired (diabetic) insulin secretion to that of normal young rats. Further, preliminary measurements of the rates of proliferation of β-cells in response to the GLP-1 infusions, using immunostaining of the pancreas with an antiserum to proliferating nuclear antigen (PCNA), suggests a stimulation of proliferation. These preliminary findings suggest the possibility that the long-term administration of GLP-1 may stimulate β-cells neogenesis and/or proliferation and raise the further possibility that the expression of the homeodomain protein IDX-1 or the E47 proteins may be upregulated to account for the increase in insulin production. Much more experimentation is required to support these preliminary findings (See D. Experimental Design and Methods). However, it is tempting to speculate that an eventual long-term treatment of diabetic subjects with GLP-1 may not only stimulate insulin secretion and peripheral glucose utilization, suppress glucagon secretion and hepatic glucose output, but also enhance the formation of β-cells.

12th International Symposium on Regulatory Peptides

Type name, address, and telephone number of authors who should receive correspondence in area C and complete areas A and B. Please be sure to check the appropriate category for your abstract in area B.

Signature of Principal Author

☐ Check if abstract is submitted for the Young Investigator Travel Award

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☒ ...velopment
☐ ...l Transduction/Kinase/Phosphatase
☐ ...Biosynthesis
☐ ...cal
☐ ...ction
☐ ...Regulation of Genes
☐ ...Nitric Oxide

Abstract Deadline March 1, 1998

GLP-1 IS A TROPHIC FACTOR.
J. M. Egan, J. Zhou, R. Perfetti, D. Elahi,
NIA, Baltimore, MD.; MGH, Boston, MA.

Glucagon-like-peptide-1 (GLP-1) is a potential candidate for the treatment of type 2 diabetics because it normalizes blood glucose levels. We have shown that GLP-1 increases intraislet insulin content in Wistar rats. This is accompanied by an increase in mRNA of insulin, glucokinase and GLUT2 transporter. There is a 26% increase in pancreatic weight with 5 days of chronic subcutaneous treatment with GLP-1. To examine whether an increase in cell turnover occurs during treatment with GLP-1 or a potent analog, exendin-4, we used proliferation cell nuclear antigen (PCNA) as a marker for proliferation of cells. An increase in PCNA in the acinar portion of the pancreas and in the progenitor pancreatic cells in the ducts was observed. Furthermore there was a progression with the greatest density in PCNA positivity observed in the smallest ducts to the lowest density in the largest ducts. Two days of treatment with GLP-1 ($1.5 pmol.kg^{-1}.min^{1}$) or daily injection of exendin-4 (0.5 nmol/kg, IP) resulted in glucagon containing cells in the ducts. After 6 days of treatment, positive staining for insulin was observed in the smallest ducts as well as in small nests of cells within the acinar portion of the gland. We conclude that GLP-1/exendin-4, similar to what has been shown for GLP-2 in the intestine, causes differentiation of progenitor pancreatic cells into cells containing glucagon and insulin.

I ... Elahi
(Laboratory Director) ... certify that Jie Zhou ... is eligible for the Young Investigator Travel Award.

C.

Principal Author's Name: **Dariush Elahi**

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Telephone: **(617) 724-0955**

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EXHIBIT E

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TO: DAVID GLASS

FROM: JOEL HABENER

DATE: 10/28/91

FAX NO: 6-1668

NO. of Pages: 7
(including this page)

David Hi,

These may need to be revisited. Some of the data have been generated with the Novo Nordisk analog. Other data by/with NIH collaborators.

Joel

Please call if entire transmission is not received or if it is routed incorrectly. Thank you.

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To: MARIE LOSSKY   From: JOEL HABENER

Fax: 6-1668   Date: 10/19/00

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Hi!

This is the original disclosure —
Communications. The new findings
are that the progenitor cells are the nestin-positive
islet-derived progenitor stem cells.

GLP-1 → Stem Cells → β-cells
(islet derived)
(? IDX-1)

Joel

LABORATORY OF MOLECULAR ENDOCRINOLOGY
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To: MARIE LOSSKY   From: JOEL HABENER

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•Comments:

Hi!

This is the original disclosure — Communications. The New findings are that the progenitor cells are the nestin-positive islet-derived progenitor stem cells.

GLP-1 → *islet derived* Stem Cells → β-cells
(↑ IDX-1)

Joel

From: Lossky, Marie

To: Habener, Joel F

Cc:

Subject: RE: New patent

Sent: 10/18/00 8:04 AM   **Importance:** Normal

Joel,

Thanks for the fax. I'll have to get Marv involved in positioning IP protection around this so as not to throw off any of your existing IP portfolios. It would be helpful to us if you would fill out the attached invention disclosure form. In particular, what experimental evidence do you have to support the hypothesis? How does the hypothesis fit in with prevailing thinking in the field?

Also, could you please expand/comment on your proposed use of the GI mice? You mentioned in passing yesterday that you wanted to use these animals in some of your stem cell work, and I am not sure which of the following statements (from Exhibit A, the Research authorized under the Material Transfer Agreement with GI) would be relevant:

    1. Analyze GFP expresion during embryonic development and   correlate the findings with results from immunohistochemical analyses of PDX protein levels in tissue sections from the same   mice.

    2. Use the PDX/GFP and INS/LacZ transgenic mice in stides   to determine the effects of islet growth factors, such as GLP-1,   on PDX and insulin gene expression in adult mice.

    3. Look for GFP expression in brain sections from PDX/GFP   transgenic mice to corroborate and extend intitial findings of PDX expression in mouse brain.

I should remind you that any patentable inventions or unpatentable results that you obtain using these animals are obligated to GI's parent company, American Home Products. This may not be an opportune time to enter into agreements that tie up pieces of your stem cell story, but of course the decision to do that is ultimately up to you.

Thanks.
Marie

Invention Disclosure Form.ID+NCD.5.00.doc

Marie Lossky, Ph.D.

Industry Agreement Associate
Corporate Sponsored Research and Licensing,
Massachusetts General Hospital
tel: (617) 726-8629
fax: (617) 726-1668

-----Original Message-----

    From: Habener, Joel F
    Sent: Tuesday, October 17, 2000 6:35 PM
    To: Lossky, Marie
    Subject: New patent

    Hi!
        I just faxed the model and the idea of linking the various components of the idea together.
    Joel

MGH INVENTION DISCLOSURE FORM

1. ## TITLE OF INVENTION:

> Stimulation of IDX-1 expression and β-cell neogenesis by GLP-1

2. ## INVENTOR(S) NAME, TITLE, LAB, DEPT., AND TEL. EXT.:

Joel F. Habener, MD
Professor of Medicine
Laboratory of Molecular Endocrinology   6 6950

3. ## SOURCE OF FUNDS FOR THE RESEARCH WHICH RESULTED IN THE INVENTION:

A.		Government Grant - Agency and Grant No.	DK 30834, DK 30457
B.		Private Industry - Name	
C.		MGH	
D.		Foundation	
E.		Other - Explain	Howard Hughes Medical Institute

4. ## INVENTION DISCLOSURE: Describe the invention in sufficient detail, using the outline below to convey a clear understanding of the nature, purpose, operation and the physical, chemical biological or electrical characteristics of the invention. Attach sketches, drawings, photos, diagrams or photos, and any pertinent manuscript which described the invention:

A.   State in general terms the purpose and object of the invention.

B.   Describe the background of the invention and how the invention overcomes problems that existed previously.

C. Describe the invention in detail, particularly pointing out novel features and critical components. Include sketches, drawings, circuit diagrams. If the invention relates to a new composition of matter, give the structural formulas for all novel compounds, the process for synthesizing or isolating the compounds, all available chemical and physical properties and all test data which show the utility and efficacy of the compounds. A copy or manuscript of a draft including this information will usually be acceptable.

5. **PUBLICATION, SALE OR USE OF THE INVENTION:**

A. Have you described your invention in a publication?

| YES | | NO | X |

B. If YES, give name and date of publication.

C. If NO, what plans do you have for publication in the future?

Prepare a manuscript in 1-2 months

D. Has your invention been used? When and under what circumstances?

GLP-1 is under development by NOVO Nordisk to treat type 2 diabetic subjects

E. Has your invention been offered for sale? When and under what circumstances?

No

2

6. **COMMERCIAL POSSIBILITIES:** To the extent know, please state whether the invention has significant commercial potential. Is the invention primarily a research tool? How extensively will it be used by the public? Does it appear to have significant commercial potential outside the United States?

> It is potentially useful for treatment of
> diabetes mellitus, estimated 100 million individuals
> world-wide

7. **HISTORY OF THE INVENTION:**

   A. When did you first think of (conceive) the invention?

Date	6/85

   B. When did you first disclose your invention to another person?

Date	6/85	To Whom	Heather Hermann

   C. When was the first written description or drawing of your invention produced? Please attach photocopy of such written description.

Date	NA

8. **INTERACTIONS WITH THIRD PARTIES:**

   A. Have you or any co-inventor(s) listed in (2) above received Biological Materials from any industrial or academic source for use in the research which gave rise to the invention? If so, please list the Material and attach a copy of each such Agreement.

   > No

(3)

B.   Have you or any co-inventor(s) listed in (2) above entered into or signed a confidentiality or secrecy Agreement in exchange for receiving any proprietary information from a third party pertaining to the research which gave rise to the invention? If so, please describe briefly the subject or the confidentiality or secrecy Agreement (s) and attach a copy of each such Agreement.

No

INVENTOR(S)' SIGNATURE(S):

DATE

Joel F. Habener   10/28/97

WITNESS(ES): _Disclosed to and understood by me on:_

DATE   SIGNATURE

④

4. Diabetes Mellitus affects approximately 16 million people in the USA (100 million world-wide). Individuals with type 1 diabetes have lost their ability to produce insulin due to the immune destruction of their pancreatic β-cells, which secrete insulin. Individuals with type 2 diabetes have lost their ability to over-produce insulin to maintain euglycemia in the presence of insulin resistance. In both types of diabetes there is a marked reduction in the mass of β-cells in the pancreas.

It is believed that the endocrine pancreas (β-cells) are derived from progenitor cells in the ducts of the exocrine portion of the pancreas. Transcription factors have been identified that are involved in pancreatic development and the stimulation of insulin gene transcription. The expression of transcription factors is believed to be regulated by growth factors, otherwise known as hormones or morphogens. Glucagon-like peptide-1 is an intestinal hormone that is released in response to feeding and stimulates the β-cells to make and secrete insulin. GLP-1 is under development as a promising potential treatment for type 2 diabetes, because it stimulates the pancreas to make its own insulin and it does not over-stimulate insulin secretion because its actions shut off when the blood sugar drops to dangerous levels. In addition, GLP-1 is now known to control appetite and to induce individuals to loose weight. GLP-1 also augments insulin mediated uptake of glucose by the liver, skeletal muscle and adipose tissue, thereby improving insulin sensitivity.

Now we find that GLP-1 stimulates the growth of new β-cells, the neogenesis of β-cells derived from the progenitor cells located in the ducts of the exocrine pancreas. GLP-1 also stimulates the expression of the transcription factor IDX-1 that appears to be responsible for the initial differentiation of precursors into β-cells and to regulate the expression of the insulin gene.

Thus, we now have evidence that GLP-1 stimulates both the formation of new β-cells and stimulates existing β-cells to grow.

Based on this new evidence, GLP-1 holds promise as a treatment for both type 1 and type 2 diabetes, because in both types of diabetes the progenitor cells in the ducts are unaffected and can be encouraged to develop and grow, and to restore the loss of β-cells mass by the administration of GLP-1.

We have patents on GLP-1 and IDX-1, but it is unclear whether they address this newly discovered property of GLP-1 to induce IDX-1 and thereby to stimulate the growth of β-cells and the production of insulin.

Massachusetts General Hospital

Date: 18-Nov-1997 11:03am GMT
From: Glass, David J.
          Glass.David@MGH.HARVARD.EDU@I()
Dept:
Tel No:

TO: Habener, Joel F   (HABENERJ@A1)

Subject: New Invention (New reference MGH 1277.0)

Joel:

I've reviewed the new invention you sent me at the end of October. I
agree that it seems to overlap somewhat with your earlier patents, but
it is not clear whether this new use would be patentable with respect to
the earlier patents. I'm happy to send the new invention to one of our
attorneys to answer this question, but I think there is a different
threshold question. The new invention is a new use for GLP-1, and as
such, can only be practiced by someone licensed under our GLP-1 patents
(is this correct?). If so, that means we effectively have only one
potential licensee for this new invention, the Scios/Novo "team", and we
should contact them about this new use before going too far down the
road. Have you told anyone at either company about this new finding?

Let's discuss how best to proceed. Thanks.

David
------------------------------- ATTACHMENT -------------------------------

Massachusetts General Hospital
13th Street, Building 149, Suite 1101
Charlestown, MA 02129
Tel.: 617-726-8608
Fax.: 617-726-1668

To: DR. Joel Habener **Fax:** 6-6954

Company:

CC: **Fax:**

From: David Glass **Date:** 7/1/98

Re: New Invention Disclosure

Page(s): including cover sheet 2

☐ **Urgent**   ☐ **For Review**   ☐ **Please Comment**   ☐ **Please Reply**

● **Comments:**

The Massachusetts General Hospital

Thirteenth Street, Building 149, Suite 1101

Charlestown, Ma 02129

DAVID J. GLASS, Ph.D.
Associate Director for Patents
Office of Technology Affairs

Telephone: (617) 726-5474
Telefax: (617) 726-1668
E-mail: glass@helix.mgh.harvard.edu

July 1, 1998   **BY FACSIMILE**

Michele Cimbala, Esq.
Sterne, Kessler, Goldstein and Fox
1100 New York Avenue, Suite 600
Washington, DC 20005-3934

Re: New Invention Disclosure
       Title: Stimulation of IDX-1 Expression and β-Cell Neogenesis by GLP-1
       Inventor: Joel Habener, M.D.
       MGH Ref: 1277.0

Dear Michele:

Enclosed please find the above-captioned invention disclosure from Dr. Joel Habener, along with some e-mail correspondence from last fall and this week describing the invention and some current research of Dr. Habener's and others' that may be relevant thereto.

It is not clear to me from this correspondence exactly what has been published by other groups (Dr. Habener has evidently not yet published his own research). However, Dr. Habener would like to explore the possibility of filing a new patent application on this invention, either as a stand-alone patent application or as a CIP (or even a continuation or divisional?) to the MGH 213 series of cases that is still pending. Please briefly review this material, talk to Dr. Habener as may be necessary, and let me know what you think our options are with respect to a possible new patent filing. It is not necessary to do a search at this time, although a major concern for me is the impact of our prior patent applications on the patentability of this invention, and whether this might require that we claim priority from these earlier cases.

Thank you very much. Please let me know if you have any questions.

Sincerely,

David J. Glass, Ph.D.

Enclosure
cc: Joel Habener, M.D.

MASSACHUSETTS
GENERAL HOSPITAL

HARVARD
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Joel F. Habener, M.D.
Professor of Medicine
Harvard Medical School
Chief, Laboratory of Molecular Endo...
Massachusetts General Hospital

Tuesday, April 18, 2000

C O P Y

Marvin C. Guthrie, JD
Executive Director for Patents and Licensing
Office of Corporate Sponsored Research and Licensing
CNY 149.1101

RE : GLP-1

Dear Marvin:

Since the initial submission to you of the extended invention disclosure (October 28, 1997, enclosed), there has been substantial new discovery on the actions of GLP-1 agonists to promote the growth of new pancreatic β-cells and thereby to promote the production of insulin, relevant to the treatment of individuals who have diabetes mellitus due to a failure of the pancreas to produce insulin in amounts sufficient to meet the body's needs.

The novel findings are that GLP-1 stimulates pancreatic stem cells to differentiate into insulin-producing β-cells and does so by stimulating the expression of the homeodomain transcription factor IDX-1 (also known as IPF-1 and PDX-1). GLP-1 is in development for the treatment of diabetes by Novo Nordisk, Eli Lilly, Glaxo Welcome, Novatis, and Bayer. The first generation GLP-1 drugs are scheduled for marketing in late 2002 – early 2003. The MGH holds 4 – 5 patents on GLP-1 for the treatment of diabetes, all of which expire in 2003, 17 years since the filing of the parent application in 1986. This is the time to file a new patent to extend coverage for GLP-1. The estimated worldwide sales of GLP-1 in 2003 are $0.5 – 3.0 billion, depending upon market penetrance to displace the use of insulin. This could represent considerable royalty income to the MGH, if the MGH is appropriately positioned with regard to patent rights.

At present, Stern, Kessler, Goldstein and Fox are dealing with the GLP-1 patents. Banner Witkoff is dealing with the patents on IDX-1 and stem cells. I propose that the OTA devise a strategy to combine these efforts to construct a new patent to propose GLP-1 stimulates pancreatic stem cells to differentiate into insulin-producing β-cells and does so by stimulating the expression of the β-cell-specific master regulator of pancreas development and of insulin gene transcription, IDX-1.

PARTNERS Health Care System Member

The new data relevant to the construction of a new patent application are:

1.   GLP-1 agonists stimulate the formation of new β-cells in the rat *in vivo* and prevent the occurrence of diabetes in the rat model of type 2 diabetes of partial pancreatectomy (Xu et al., Diabetes 48:270,1999). GLP-1 agonists stimulate the expression of IDX-1 (Stoffers et al., Diabetes, in press, May 2000). Pancreatic islets contain pancreatic stem cells (Diabetes, in revision). Pancreatic duct cells contain stem cells that can become β-cells (Peck et al., Nature Medicine, April 2000). Delivery of IDX-1 to liver converts hepatic stem cells into β-cells that produce insulin (Ferber et al., Nature Medicine, May 2000). GLP-1 converts transformed human fetal islet cells into β-cells (Levine et al., in press).

Much of this new fast-breaking information is in press. It is critically important to file a new patent application on GLP-1. I hope to receive a response from OTA ASAP.

Sincerely yours,

Joel F. Habener, M.D.

cc: David J. Glass, John T. Potts, Jr.

JFH/ral

EXHIBIT F

[^{3}H]TdR Labeling of Islet Cells #2

Target cells

~~RIN 1046-38 cells~~ βTC-1 Cells

~~HIT cells~~ αTEC cells

Duplicates of everything

Really confluent and not so confluent

A. Conditioned media

~~RIN 1056A~~

HAM INRIG9 - 24 hr. media from relatively confluent plates

Run target cells overnight in 0.25% BSA and duplicate wells of cells in FBS (regular) media

Remove media and add either:

1. Conditioned media
2. Normal fresh FBS media
3. 0.25% BSA media
   24 hours later add:
   0.1 mCI (0.1 ml) ^{3}H TdR/ml
   ? 0.05 mCI (0.05 ml) for 1 hr.

B. GILS's

GLP-I(7-37)10^{-7}M
GLP-II(1-33)10^{-7}M
Run target cells overnight in 0.25% BSA and duplicate wells of cells in FBS media

Remove media

Add back BSA media with:

1. Nothing
2. GLP-I(7-37)10^{-7}M
3. GLP-II(1-33)10^{-7}M
4. + regular media (No BSA)

~~At least 12~~ About 24 hours later add 0.1 mCi/ml [^{3}H]TdR for 1 hr.

GLP-1 (7-37) $10^{-7}M$ vs 0

Setup on Slides:
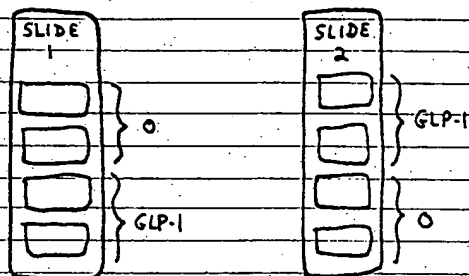
Slides plated out 1ml/well at a 1→4 dilution on 7/12/87. Plastic slides - 4 well ea. RIN 38 F_3

7/16/87 2:00pm - medium changed to 0.5% FBS to slow growth

7/17/87 8 am - Transfered cells to Medium with 0.25% BSA (no FBS) with/without GLP-1 (7-37) at $10^{-7}M$

   2:00pm - Added ^3H TdR If 1mCi/ml, add 200 λ/well (removed 200λ medium first)
   (The way it comes). 400 λ / big well

   3:00 pm - Drained wells by upending on a blue pad, then peeled off wells and washed cells in fresh PBS.

   3:15 pm - Cells float off slides in whole sheets. Attempt to save by fixing with 4% PFA. Total mess.

Revised Strategy: Grow on glass slides?

    Wash cells and fix before ripping off wells.

    Fix before washing?

3H-TdR LABELING OF 1046-38 CELLS

1. Grow cells on slides:

    3 control--media alone   changed 9/22/87 JFH

    3 experimental-GLP-I(7-37) 10-8 M

    2 experimental-GLP-I(7-37) 10-7 M

2. Transfer to media containing 0.5% FBS and grow (incubate) for 24 hrs.

3. Add GLP-I(7-37) at 0, 10-8, 10-7 M in bioassay buffer), 0.25% BSA.

4. Incubate 6-8 hours.

5. Add 3H-TdR ca. 0.1 mCi per 1-2 ml.   Incubate 1 hr.

6. Remove 3H media, wash wells well, and fix for autoradiography.

11/9/87 11 pm   Took 2 plates (100mm) HIT F_{18} and   2 plates Jan's 1046-38 F_6 and pooled each set → 50~

     I created   2   4 well plastic slides   at 1→25 dilution   1ml/well

           2   "   "   1→80 dilution   "

           2   4 well glass slides   1→2.5 ml   "

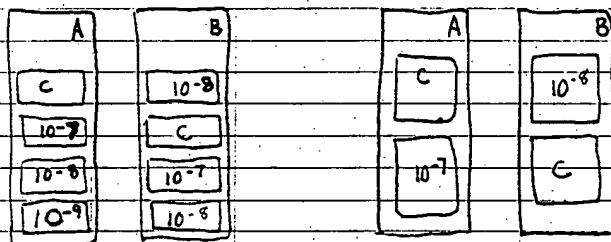   2   "   "   1→10   "

           2   2 well plastic slides   1→2.5   2ml/well

           2   "   "   1→10   "

           2   2 well glass slides   1→2.5   "

           2   "   "   1→10   "

11/11/87   Changed all media to   0.5% FBS to slow growth   —   Total volume = 64 ml DMEM
    10 am   64 ml RPMI

11/12/87   Made up GLP-1 solutions (7-37) and added to cells.
    4 pm   (all in 0.25% BSA + Medium)

                                       40 ml Medium 0.25% BSA

	A	B		A	B
	C	10^{-8}		C	10^{-8}
	10^{-7}	C			
	10^{-8}	10^{-7}		10^{-7}	C
	10^{-9}	10^{-8}			

                                                        Totals Needed

2 ml   Control   × 4 = 8   4 ml Control   × 4 = 16   24 ml Control

2 ml 10^{-7} 8   2 ml 10^{-7} 8   16 ml 10^{-7}

3 ml 10^{-8} 12   2 ml 10^{-8} 8   20 ml 10^{-8}

1 ml 10^{-9} 4   4 ml 10^{-9}

                                                     Need half in each medium

Dilution Scheme: 0.5ml H_2O to tube = 10^{-5}

               500λ   1.25ml   500λ

             500λ   10ml   125ml   5ml
             10^{-5} 10^{-7} 10^{-8} 10^{-9}
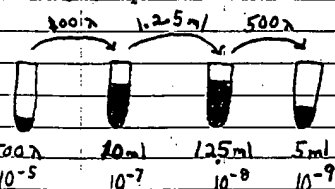
Sort of botched makeup of solutions, i.e. ran out so had to make more. Should all be the same, as I was compulsive about it. Put only one ml on 2-well plate wells. 1046-38 cells went first, so ironed the glitches for the HIT cells

11/12/87   Had limited Thymidine Resources so added to best guess pairs first — ie plastic 4-well 1→10 dilution slides.
   11:00 pm   Saved medium and used on other slides.   1 hr incubation.

         Thymidine comes 1mCi/1ml aqueous solution, so added 100λ/well for ~0.1mCi/ml. Swished up pipette to mix.

   12:30   Put ^{3}H TdR medium on plastic 4-well 1→2 dilution slides. (Washed first set in PBS 3 × 10 min. 4% PFA 10 min.)
   2:00   Split medium in half — did both sets glass 4-well slides (1→2 and 1→10 dilution 500λ each — figured concentration counts

2:00 am -   Generalized mess.

1046-38 cells on first batch of slides mostly vanished. HIT cells clung beautifully.
        (The 4-well plastic 1→10 dilution slides) Rinsed with H_2O + dried. Then peeled off wells.

So, on the 2nd batch of slides, I was extremely careful (4-well plastic 1→2.5 dilution).
    I decided to go for the 4% PFA after 2 quick PBS washes. Instantaneously, the
    the 1046-38 cells lifted off in sheets. So I carefully removed the 4% PFA and rinsed
    only with several drops of H_2O and drained and air dried. I saved some sheets, but
    the background may be terrible. Preservation looks OK so far. I figure each slide is
    internally controlled, so if I at least do the same thing to each well in a slide, I
    can vary a bit between slides. HIT clung beautifully, even though very confluent.

The third batch (all the 4-well glass slides) "A" slides got their peptide medium saved (in case
we want to check GLP-1 concentrations). Then I put ½ ml of the hot medium in each well so
I could do both the 1→2.5 and 1→10 dilution slides at once. Need to save time.
    I tried the quickie 4% PFA fix after a fairly long PBS soak session (as long as cells stayed
on I figured I should keep rinsing. But then after fixing, the cells were loose and when the
slides dried (1046-38) the cell slush gathered in the middle of the slide.
                                                                            (1046-38)
The 4th batch (glass 2-well 1→10 dilution slides) I thought I would try not fixing. When I
rinsed quickly with H_2O, the cells plumped up and popped and what was left fell off the slide. When
I dried directly from PBS, the salt made them shrivel. I fixed the HIT cells very quickly in 4% PFA
and rinsed with H_2O. Some not fixed enough popped.

The 5th batch (glass 2-well 1→2.5 dilution slides) I thought I'd try acetone fixation (it dissolves plastic).
It precipitated the salt into a white wasteland.

9:00 am   The remaining slides I was too damn tired to deal with so I let them rot. | There were 4:
            1046-38 1→2 A 2 well plastic | (no great loss)
            1046-38 1→2 B 2 well plastic | tossed later.
            HIT 1→2 A 2 well plastic
            HIT 1→2 B 2 well glass ← previously graphed wrong slide.

Conclusions :   Rinsing before peeling wells off is a good idea. Can at least contain loose sheets of cells and
            have a fighting chance to save same. Doesn't prevent 1046-38 cells from coming off in the first place.
            The 1046-38 cells seem to stick a tad better on glass

^3H TdR Experiments

① Further attempts to slow cells down
② Further pursuit of "conditioned" media effect

① Try growing βTC·1 & IEC-18 cells
in 1% FBS for 24 & 48 hrs followed
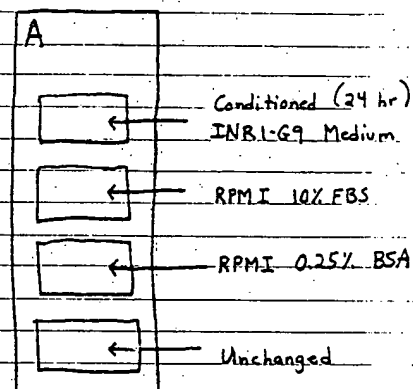by 1 hr pulse label with ^3H TdR
For control use 10% FBS media

② Repeat conditioned media Exp's using   on βTC-1 cells
Media from INR1G9 again
and media from αTC-1 cells.
and media from βTC-1 cells (homologous media

Experiment A: Conditioned Media

Slide #	Cell Type	Confluency	RPMI 1st Night	2nd Night (24 hrs)
1, 2	β TC-1	full	0.25% BSA	
3, 4		½	↓	
5, 6		full	10% FBS	
7, 8		½	↓	
9, 10	IEC-18	full	0.25% BSA	
11, 12		½	↓	
13, 14		full	10% FBS	
15, 16		½	↓	

A
- ← Conditioned (24 hr) INR1-G9 Medium
- ← RPMI 10% FBS
- ← RPMI 0.25% BSA
- ← Unchanged

Experiment B: GLP's

Slide #	Cell Type	Confluency	RPMI 1st Night	2nd Night (24 hrs)
17, 18	β TC-1	full	0.25% BSA	
19, 20		½	↓	
21, 22		full	10% FBS	
23, 24		½	↓	
25, 26	IEC-18	full	0.25% BSA	
27, 28		½	↓	
29, 30		full	10% FBS	
31, 32		½	↓	

B
- ← 0.25% BSA
- ← 0.25% BSA + GLP-1 (7-37) 10^{-7} M
- ← 0.25% BSA + GLP-II (1-33) 10^{-7} M
- ← RPMI 10% FBS
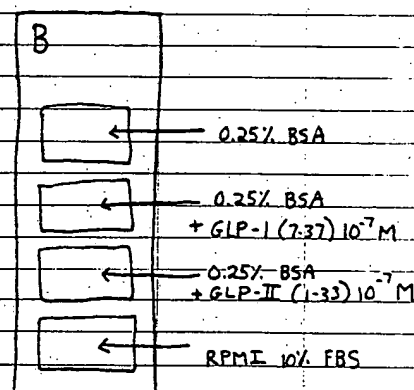
Solutions: 500 ml RPMI ($^2/_{25}$)

              add 5 ml P/S

    200 ml   300 ml

    add 0.5g BSA   add 30 ml FBS

    filter 0.22μ   filter 0.22μ ⟶ don't need to do all, just 200 ml or so.

                 (Use also on INR1-G9 cells for conditioning)

GLP-1 (7-37) 10^{-7}M : Add 0.5 ml H_2O to tube to get 10^{-5}M. Take 200λ → 20 ml BSA medium = 10^{-7}M

                        ~~Take 200λ → 20 ml FBS medium = 10^{-7}M~~

GLP-II (1-33) 10^{-7}M : Add 50λ 10% HAc to tube. Dissolve. Add 450λ H_2O. Take 200λ → 20 ml BSA medium. pH $\bar{\text{c}}$ NaOH = 10^{-7}

                        ~~Take 200λ → 20 ml FBS medium - pH $\bar{\text{c}}$ NaOH = 10^{-7}M~~

4/10/88   9-10pm changed slides from BSA or FBS RPMI to test medium. Most slides totally confluent or over confluent. So much for worrying they wouldn't be confluent enough.

4/11/88   11 pm   Added ^{3}H TdR   (50λ/ml of a 1mCi/ml = 0.05 mCi/ml = OK by Joel)   32 slides × 4 wells = 128 × 50λ

                                                   6.4 ml

                           (16 slides × 4 wells) + (16 slides × 3 wells) = 5.6 ml

    Concentration is what counts. ~~As~~ Remove 0.5 ml from each well, add 25λ ^{3}H TdR to what's left. Shake well. (Irritating habit of medium getting sucked out of end wells by capillary action by cover) Did 5min/8 slides

    After 1 hr, quickly drained wells with repeater pipette and pipetted in PBS. (cell) 0.5ml (Did 8 slides × 4 racks in 20 min) Then drained PBS and added PBS + 4% PFA (0.5ml) after 20 min. After PFA for 20 min, slides were drained and dismembered. Slides were washed 20 min in 3X changes ICC PBS, then briefly dipped in H_2O to desalt before air drying. Of course morphology declined upon drying, but I didn't lose cells.

4/15/88   Did 6 hr exposure of odd slides with Ultrafilm. 4min dev. 5min fix. 30 min wash. Air dry. Looked OK

4/17/88   Did 12 hr NTB-2 (1:1 H_2O) dip of Slide #1. 4min dev, 5 min fix, 30min wash, 2½ min counterstain, glycergel mount. Perfect.

H

On re thinking it would be good to run the βTC-1 cells in **No** Serum & another batch in 1% Serum for 48 hrs to slow them down.
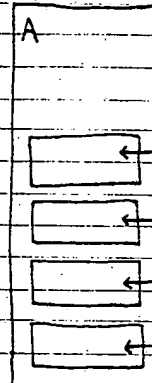
Experiment A: Slowing Cell Growth

Slide #	Cell Type	Confluency	Time
1,2	β TC-1	High	48 hrs
3,4		Medium	
5,6		Low	
7,8		High	24 hrs
9,10		Medium	
11,12		Low	
13,14	IEC-18	High	48 hrs
15,16		Medium	
17,18		Low	
19,20		High	24 hrs
21,22		Medium	
23,24		Low	

A

← — RPMI 0% FBS
← — RPMI 1% FBS
← — RPMI 10% FBS
← — RPMI 0.25% BSA

5/15 Sunday night: Plated out all the slides.
   βTC-1: 2½ 150mm Semiconfluent → 43 ml → 6 slides 1ml/well.
   Then 6 slides ½ml/well + ¼ml medium, doubled vol. left → ½ m
   IEC-18: 3/10 100mm plate → 30 ml → 4 slides 1ml/well + 4 slides ½ ml/well
   Then doubled remaining volume → 4 slides ½ ml/well

Tuesday Noon: Washed 48 hr slides 2X w/ 0% FBS, then set up, ¾ml/we
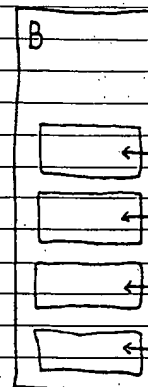   Put medium on donor plates - ∝, β cells 150mm, INRI G9 100m

Wednesday Noon: Washed + Set up rest of slides. ¾ml/well. Didn't spin donor medium
       (removed 35c

Thursday Noon: Added ^{3}H TdR. (Added 500 λ H₂O to 2.1ml left) 20λ/well in 400
       in NEN vial.

Experiment B: The Conditioned Medium Effect

Let sit 1 hr (±3min). Removed medium.
Washed 1X w̄ PBS, fixed 20min 4% PFA/PB
Rinsed all slides 1x PBS, then in 1.5L 2hr
H₂O dip, then air dry.

B

Slide #	Cell Type	Confluency	Donor Confluency
25	β TC-1	High	High
26		Medium	
27		Low	
28		High	Low
29		Medium	
30		Low	

← RPMI from INRI-G9 (24hr)
← RPMI from ∝ TC-1 (24hr)
← RPMI from β TC-1 (24hr)
← Fresh RPMI (10% FBS as above)

Films: Ultrafilm 9 hrs (forgot) = too long.

Making Solutions for ^3H TdR

Needed:

24 ml	RPMI 0% FBS	+ 120 ml to Wash everything	→ Say 400 ml
24 ml	RPMI 1% FBS		→ Say 50 ml
24 ml	RPMI 10% FBS	+ 105 ml for 24 hr collection	→ Say 500 ml
24 ml	RPMI 0.25% BSA	+ 24 ml for Exp. B	→ Say 50 ml
3 ml	RPMI from confluent INRI-69		
3 ml	RPMI from semi-confluent INRI-69		
3 ml	RPMI from confluent ∝ TC-1		
3 ml	RPMI from semi-confluent ∝ TC-1		
3 ml	RPMI from confluent β TC-1		
3 ml	RPMI from semi-confluent β TC-1		

= 2 bottles
filter sterilize everything.
(Includes Pen/Strep)
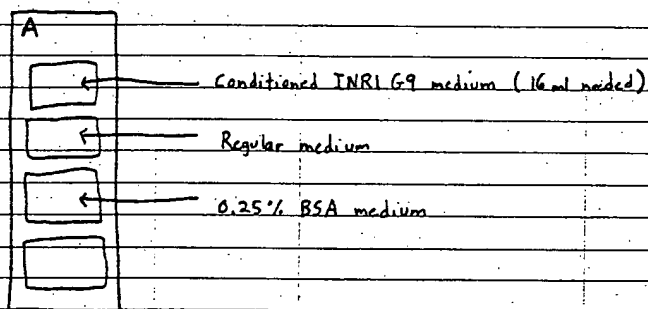
1. Grow up INR1-G9 cells to use medium (RPMI + 10% FBS)

2. Grow up 16 ♦ 4-well glass slides each of βTC-1 cells (DMEM 15% HS, 2.5% FBS) (can use RPMI 10% FBS)
                                                   IEC cells (RPMI 10% FBS)
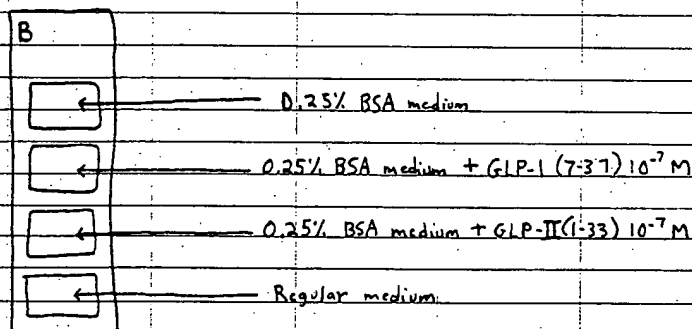
        Half the slides should be confluent, half semi confluent (ie 8 each)
        1 ml medium/well

3. Change the medium. Replace half the slides with regular medium, half with 0.25% BSA medium.

4. Let incubate overnight

5. Remove medium from wells. Divide slides into 2 identical piles

        Replace medium from half the slides as follows:

```
A
  ┌──────┐ ←———— Conditioned INR1 G9 medium ( 16 ml needed)
  ┌──────┐ ←———— Regular medium
  ┌──────┐ ←———— 0.25% BSA medium
  ┌──────┐
```

        Replace medium from 2nd half of slides as follows:

```
B
  ┌──────┐ ←———— 0.25% BSA medium
  ┌──────┐ ←———— 0.25% BSA medium + GLP-1 (7-37) 10$^{-7}$ M
  ┌──────┐ ←———— 0.25% BSA medium + GLP-II (1-33) 10$^{-7}$ M
  ┌──────┐ ←———— Regular medium
```

6. After 24 hrs incubation, add 50 λ containing 0.1 mCi ^{3}H TdR to each well (in H$_2$O). Mix with P-1000 pipette.

7. Incubate 1 hr 37°C.

8. Rinse wells several times with PBS. (As much as you can stand without losing too many cells.)

9. Add 4% PFA in PBS to each well. Let sit 16 min (stagger slides 1min intervals). Rinse in PBS, then peel off wells, quick H$_2$O dip, air dry.